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(54) Title: SPECIFIC BINDING MEMBERS FOR HUMAN TRANSFORMING GROWTH FACTOR BETA; MATERIALS AND METHODS

(57) Abstract

Specific binding members comprising human antibody antigen binding domains specific for human transforming growth factor beta (TGFβ) bind specifically isoforms TGFβ2 and TGFβ1 or both, preferentially compared with TGFβ3. Specific binding members may be isolated and utilised in the treatment of disease, particularly fibrotic disease and also immune/inflammatory diseases. Therapeutic utility is demonstrated using *in vitro* and *in vivo* models. Full sequence and binding information is provided, including epitope sequence information for a particularly advantageous specific binding member which binds the active form of TGFβ2, neutralising its activity, but does not bind the latent form.

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SPECIFIC BINDING MEMBERS FOR HUMAN TRANSFORMING

GROWTH FACTOR BETA; MATERIALS AND METHODS

This invention relates to specific binding
members for human transforming growth factor beta
5   (TGFβ) and materials and methods relating thereto. In
particular, it relates to specific binding members
comprising antibody binding domains; for example,
human antibodies. Human antibodies against human TGFβ
may be isolated and utilised in the treatment of
10   disease, particularly fibrotic disease and also
immune/inflammatory diseases. The isolation of
antiself antibodies from antibody segment repertoires
displayed on phage has been described (A.D.Griffiths
et al. EMBO J. 12, 725-734, 1993; A. Nissim et al.
15   EMBO J. 13, 692-698, 1994; A.D. Griffiths et al. 13,
3245-3260, 1994; C.Barbas et al. Proc. Natl. Acad.
Sci. USA 90, 10003-10007 1993; WO93/11236). However,
the present invention provides specific antibodies
against a particular isoforms of TGFβ, which
20   antibodies have unexpected and advantageous
properties.

TGFβ is a cytokine known to be involved in many
cellular processes such as cell proliferation and
differentiation, embryonic development, extracellular
25   matrix formation, bone development, wound healing,
hematopoiesis and immune and inflammatory
responses(A.B. Roberts & M. Sporn 1990 pp419-472 in
Handbook of Experimental Pharmacology eds M.B. Sporn &

A.B. Roberts, Springer Heidelberg; J.Massague et
al.Annual Rev. Cell Biol. 6, 597-646, 1990).

The accumulation of excessive extra-cellular
matrix is associated with various fibrotic diseases.
5   Thus there is a need to control agents such as TGFβ1
and TGFβ2 to prevent their deleterious effects in such
diseases and this is one application of human
antibodies to human TGFβ.

The modulation of immune and inflammatory
10   responses by TGFbetas includes (i) inhibition of
proliferation of all T-cell subsets (ii) inhibitory
effects on proliferation and function of B lymphocytes
(iii) down-regulation of natural-killer cell activity
and the T-cell response (iv) regulation of cytokine
15   production by immune cells (v) regulation of
macrophage function and (vi) leucocyte recruitment and
activation.

A further application of antibodies to TGFβ may
be in the treatment of immune/inflammatory diseases
20   such as rheumatoid arthritis, where these functions
need to be controlled.

It is a demanding task to isolate an antibody
fragment specific for TGFβ of the same species.
Animals do not normally produce antibodies to self
25   antigens, a phenomenon called tolerance (G.J. Nossal
Science 245, 147-153, 1989). In general, vaccination
with a self antigen does not result in production of
circulating antibodies. It is therefore difficult to

raise human antibodies to human self antigens. There
are also in addition, ethical problems in vaccinating
humans. In relation to the raising of non-human
antibodies specific for TGFβ, there are a number of
5   problems. TGFβ is an immunosuppressive molecule and
further, there is strong conservation of sequence
between human and mouse TGFβ molecules. Mouse and
human TGFβ1 only differ by one amino acid residue, an
alanine (human) to serine (mouse) change at a buried
10 residue (R.Derynck et al. J.Biol. Chem. 261, 4377-
4379, 1986). Mouse and human TGFβ2 only differ at
three residues; residue 59 (T mouse, S human); residue
60 (K mouse, R human) and residue 94 (N mouse; K
human). This makes it difficult to raise antibodies
15 in mice against human TGFβ. Further, any antibodies
raised may only be directed against a restricted set
of epitopes.

    Polyclonal antibodies binding to human TGFβ1 and
human TGFβ2 against both neutralising and non-
20 neutralising epitopes have been raised in rabbit
(Danielpour et al. Growth Factors 2 61-71, 1989; A.
Roberts et al. Growth Factors 3, 277-286, 1990),
chicken (R&D Systems, Minneapolis) and turkey
(Danielpour et al. J. Cell Physiol. 138, 79-86, 1989).
25 Peptides representing partial TGFβ sequences have also
been used as immunogens to raise neutralising
polyclonal antisera in rabbits (W.A Border et al.
Nature 346, 371-374, 1990; K.C. Flanders Biochemistry

27, 739-746, 1988; K.C. Flanders *et al*, Growth Factors
3, 45-52, 1990). In addition there have been limited
reports of isolation of mouse monoclonals against
TGFβ. Following immunisation with bovine TGFβ2

5 (identical to human TGFβ2), three non-neutralising
monoclonal antibodies were isolated that are specific
for TGFβ2 and one neutralising antibody that is
specific for TGFβ1 and TGFβ2 (J.R. Dasch et al. J.
Immunol. 142, 1536-1541, 1989). In another report,

10 following immunisation with human TGFβ1, neutralising
antibodies were isolated which were either specific
for TGFβ1 or cross-reeacted with TGFβ1, TGFβ2 and
TGFβ3 (C. Lucas et al. J.Immunol. 145, 1415-1422,
1990). A neutralising mouse monoclonal antibody which

15 binds both TGFβ2 and TGFβ3 isoforms is available
commercially from Genzyme Diagnostics.

The present text discloses the first isolation of
human antibodies directed against human TGFβ1 and
against human TGFβ2. A mouse monoclonal antibody

20 directed against human TGFβ1 is available from R&D
Systems. This antibody only weakly neutralises TGFβ1
in a neutralisation assay. Neutralising mouse
monoclonal antibodies have also been generated from
mice immunised with human TGFβ1 peptides comprising

25 amino acid positions 48 to 60 (antibody reactive with
TGFβ1, TGfβ2 and TGFβ3)and amino acid positions 86-101
(antibody specific for TGFβ1; M. Hoefer & F.A. Anderer
Cancer Immunol. Immunother. 41, 302-308, 1995).

Phage antibody technology (WO92/01047;

PCT/GB92/00883; PCT/GB92/01755; WO93/11236) offers the

ability to isolate directly human antibodies against

human TGFβ. In application WO93/11236 the isolation

5   of antiself antibodies from phage display libraries

was disclosed and it was suggested that antibodies

specific for TGFβ could be isolated from phage display

libraries.

The present application shows that antibodies of

10   differing specificities for TGFβ molecules may be

isolated. TGFβ1, TGFβ2 and TGFβ3 are a closely

related group of cytokines. They are dimers

consisting of two 112 amino acid monomers joined by an

interchain disulphide bridge. TGFβ1 differs from

15   TGFβ2 by 27 mainly conservative changes and from TGFβ3

by 22 mainly conservative changes. These differences

have been related to the 3D structure (M.Schlunegger &

M.G.Grutter Nature 358, 430-434, 1992). The present

applicants have isolated antibodies which are

20   essentially specific for TGFβ1 (very low cross-

reactivity with TGFβ2); antibodies which are

essentially specific for TGFβ2 (very low cross-

reactivity TGFβ1); and antibodies which bind both

TGFβ1 and TGFβ2. Hence, these three different types

25   of antibodies, each type with distinctive binding

specificities must recognise different epitopes on the

TGFβ molecules. These antibodies have low cross-

reactivity with TGFβ3 as assessed by binding studies

6

using biosensor assays (e.g.BIACore™), ELISA and

radioreceptor assays. The most extensively studied

antibody, 6B1 IgG4, shows 9% cross-reactivity with

TGFβ3 as compared with TGFβ2, as determined by their

5   relative dissociation constants, determined using a

biosensor.

TGFβ isoforms are initially exported from cells

as inactive, latent forms (R. Pircher et al, Biochem.

Biophys. Res. Commun. 136, 30-37, 1986; L.M. Wakefield

10   et al., Growth Factors 1, 203-218, 1989). These

inactive forms are activated by proteases in plasma to

generate the active form of TGFβ. It is this active

form of TGFβ2 which binds to receptors promoting the

deposition of extracellular matrix and the other

15   biological effects of TGFβ. The active form of TGFβ

represents a relatively low proportion of TGFβ that is

in the plasma. Therefore, for a neutralising antibody

against TGFβ to be most effective at preventing

fibrosis the antibody should recognise the active but

20   not the latent form. In Example 6, it is demonstrated

that a preferred antibody of this invention ("6B1

IgG4") recognises the active but not the latent form

of TGFβ2.

The epitope of 6B1 IgG4 has been identified using

25   a combination of peptide display libraries and

inhibition studies using peptides from the region of

TGFβ2 identified from phage selected from the peptide

phage display library. This is described in Examples

11 and 14. The sequence identified from the peptide

library is RVLSL and represents amino acids 60 to 64

of TGFβ2 (Example 11). The antibody 6B1 IgG4 has also

been shown to bind to a peptide corresponding to amino

5   acids 56 to 69 of TGFβ2 (TQHSRVLSLYNTIN) with a three

amino acid (CGG) extension at the N-terminus. RVLSL

is the minimum epitope, 6B1 IgG4 is likely to bind to

further adjacent amino acids. Indeed, if the epitope

is three dimensional there may be other non-contiguous

10   sequences to which the antibody will bind. 6B1 IgG4

shows much weaker binding to the peptide corresponding

to amino acids 56 to 69 of TGFβ1 (CGG-TQYSKVLSLYNQHN).

The results of Example 14 support the assignment

of the epitope of 6B1 IgG4 on TGFβ2 to the aminoacids

15   in the region of residues 60 to 64. The peptide used

in this example, residues 56 to 69, corresponds to the

amino acids of alpha helix H3 (M.P. Schlunegger & M.G.

Grutter Nature 358 430-434, 1992; also known as the α3

helix (S. Daopin et al Proteins: Structure, Function

20   and Genetics 17 176-192, 1993). TGFβ2 forms a head-

to-tail dimer with the alpha helix H3 (also referred

to as the heel) of one subunit forming an interface

with finger regions (including residues 24 to 37 and

residues in the region of amino acids 91 to 95; also

25   referred to as fingers 1 and 2) from the other subunit

(S. Daopin et al supra). It has been proposed that

the primary structural features which interact with

the TGFβ2 receptor consist of amino acids at the C-

terminal end of the alpha helix H3 from one chain

together with residues of fingers 1 and 2 of the other

chain (D.L. Griffith et al Proc. Natl. Acad. Sci. USA

93 878-883,, 1996). The identification of an epitope

5 for 6B1 IgG4 within the alpha helix H3 of TGFβ2 is

consistent with 6B1 IgG4 preventing receptor binding

and neutralising the biological activity of TGFβ2.

As noted above if the epitope for 6B1 IgG4 is

three dimensional there may be other non-contiguous

10 amino acids to which the antibody may bind.

There is earlier advice that antibodies directed

against this region of TGFβ2 may be specific for TGFβ2

and neutralise its activity. Flanders et al

(Development 113 183-191, 1991) showed that polyclonal

15 antisera could be raised in rabbits against residues

50 to 75 of mature TGFβ2 and that these antibodies

recognised TGFβ2 but the TGFβ1 in Western blots. In

an earlier paper, K.C. Flanders et al (Biochemistry 27

739-746, 1988) showed that polyclonal antisera raised

20 in rabbits against amino acids 50 to 75 of TGFβ1 could

neutralise the biological activity of TGFβ1. The

antibody isolated in this application 6B1 IgG4 is a

human antibody directed against the amino acids in

this region which neutralises the biological activity

25 of human TGFβ2. It is surprising that such a

neutralising antibody against TGFβ2 can be isolated in

humans (where immunisation with a peptide cannot be

used for ethical reasons) directly from a phage

display antibody repertoire.

The knowledge that the residues of the alpha helix H3 form a neutralising epitope for TGFβ2 means that phage displaying neutralising antibodies are obtainable by selection from phage antibody repertoires by binding to a peptide from this region coupled to a carrier protein such as bovine serum albumin or keyhole limpet haemocyanin. This approach may be applied to select antibodies which are capable of neutralising the biological activity of TGFβ1 by selecting on the peptide TQYSKVLSLYNQHN coupled to a carrier protein. It is possible that such an approach may be extended to peptides from receptor binding regions of TGFβ isoforms, other than the H3 alpha helix.

It has further been demonstrated by the present inventors that antibodies specific for TGFβ are obtainable by isolation from libraries derived from different sources of immunoglobulin genes: from repertoires of natural immunoglobulin variable domains, e.g. from immunised or non-immunised hosts; and synthetic repertoires derived from germline V genes combined with synthetic CDR3s. The properties of these antibodies in single chain Fv and whole IgG4 format are described.

As noted above WO93/11236 suggested that human antibodies directed against human TGFβ could be isolated from phage display libraries. Herein it is

shown that the phage display libraries from which

antiself antibodies were isolated in WO93/11236 may be

utilised as a source of human antibodies specific for

particular human TGFβ isoforms. For instance, in

5   example 1 of the present application, the antibody 1A-

E5 specific for TGFβ1 and the antibodies 2A-H11 and

2A-A9 specific for TGFβ2 were isolated from the

"synthetic library" described in examples 5 to 7 of

WO93/11236 and in Nissim et al. (1994; supra). Also,

10   the phage display library derived from peripheral

blood lymphocytes (PBLs) of an unimmunised human

(examples 1 to 3 of WO93/11236) was the source for the

antibody 1B2 specific for TGFβ1. Phage display

libraries made subsequently utilising antibody genes

15   derived from human tonsils and bone marrow, have also

provided sources of antibodies specific for human

TGFβ. Thus human TGFβ is an example of a human self

antigen to which antibodies may be isolated from

"large universal libraries". Human antibodies against

20   human TGFβ with improved properties can be obtained by

chain shuffling for instance combining the VH domains

of antibodies derived from one library with the VL

domains of another library thus expanding the pool of

VL partners tested for each VH domain. For instance,

25   the antibodies 6B1, 6A5 and 6H1 specific for TGFβ2

utilise the 2A-H11 VH domain isolated from the

"synthetic library" combined with a light chain from

the PBL library.

Thus the VH and VL domains of antibodies specific

for TGFβ can be contributed from phage display

libraries derived from rearranged V genes such as

those in PBLs, tonsil and bone marrow and from V

5   domains derived from cloned germline V segments

combined with synthetic CDRs. There are also shown to

be a diverse range of antibodies which are specific

for TGFβ1 or TGFβ2. The antibodies which have been

isolated both against TGFβ1 and TGFβ2 have mainly

10   utilised V genes derived from VH germlines of the VH3

family. A wider variety of light chain variable

regions have been used, of both the lambda and kappa

types.

Individual antibodies which have been isolated

15   have unexpectedly advantageous properties. For

example, the antibodies directed against TGFβ2 (6H1,

6A5 and 6B1) have been shown to bind to TGFβ2 with

slow off-rates (off-rate constants k_{off} of the order of

10^{-3} s^{-1} and dissociation constants of less than 10^{-8}M)

20   to neutralise TGFβ2 activity in in vitro assays and to

be potent in in vivo applications. The antibody 6B1

IgG4 has been shown to bind specifically to TGFβ2 in

immunohistochemistry in mammalian tissues and not to

cross-react with other antigens in human tissues. The

25   properties of these antibodies may make them

particularly suitable for therapeutic applications.

The fact that these antibodies share the same heavy

chain, shows that VH domains can be effective with a

12

number of different light chains, although there may
be differences in potency or subtle changes of epitope
with different light chains. As shown in Examples 3
and 4 and Tables 4 and 5, 6B1 IgG4 is the most potent

5   antibody in neutralising TGFβ2 activity in the
radioreceptor assay and the TF1 proliferation assay.
Its properties may however be expected to be
qualitatively similar to the antibodies 6A5 and 6H1
with which it shares a common VH domain. Thus the

10   reduction in neural scarring observed on treatment
with 6A5 single chain Fv and 6H1 IgG4 shown in Example
5 would be expected to be reproduced with 6B1. The
antibodies directed against TGFβ1 (particularly 1B2
and its derivatives) also have unexpectedly

15   advantageous properties. Antibody 27C1/10A6 derived
from 1B2 by chain shuffling, spiking and conversion
into whole antibody IgG4 , has been shown to be potent
in an *in vitro* scarring model. The VH domain of this
antibody was derived by site directed "spiking"

20   mutagenesis from the parent antibody 7A3. A large
number of spiked clones were obtained which show
similar properties in in vitro assays. There can be a
number of changes in CDR3 of the VH compared to 27C1,
for instance, 28A-H11 differs in 7 of the 14

25   positions, 2 of which are non-conservative changes.
Thus there may be up to 50% of the residues in the VH
CDR3 changed without affecting binding properties.
        Antibodies specific for human TGFβ1 and human

TGFβ2 have been shown to be effective in animal models
for the treatment of fibrotic diseases and other
diseases such as rheumatoid arthritis where TGFβ is
overexpressed. Antibodies against TGFβ have been

5   shown to be effective in the treatment of
glomerulonephritis (W.A Border et al. Nature 346, 371-
374, 1990); neural scarring (A. Logan et al. Eur. J.
Neurosci. 6, 355-363, 1994); dermal scarring (M. Shah
et al. Lancet 339, 213-214 1992; M.Shah et al. J.Cell

10   Science 107, 1137-1157, 1994; M. Shah et al. 108, 985-
1002, 1995); lung fibrosis (S.N. Giri et al. Thorax
48, 959-966, 1993); arterial injury (Y.G. Wolf, L.M.
Rasmussen & E. Ruoslahti J. Clin. Invest. 93, 1172-
1178, 1994) and rheumatoid arthritis (Wahl et al J.

15   Exp. Medicine 177, 225-230, 1993). It has been
suggested that TGFβ3 acts antagonistically to TGFβ1
and TGFβ2 in dermal scarring (M.Shah et al. 1995
supra.). Therefore, antibodies to TGFβ1 or TGFβ2 with
apparent low cross-reactivity to TGFβ3, as assessed by

20   binding studies using a biosensor assay (e.g
BIACore™), ELISA or a radioreceptor assay, as
disclosed in this application, that is to say
antibodies which bind preferentially to TGFβ1 or TGFβ2
compared with TGFβ3, should be advantageous in this

25   and other conditions such as fibrotic conditions in
which it is desirable to counteract the fibrosis
promoting effects of TGFβ1 and TGFβ2. An antibody
which cross-reacts strongly with TGFβ3 has however had

14

an effect in an animal model of rheumatoid arthritis
(Wahl et al., 1993, supra).

There are likely to be applications further to
the above mentioned conditions, as there are several

5   other in vitro models of disease where antibodies
against TGFβ have shown promise of therapeutic
efficacy. Of particular importance may be the use of
antibodies against TGFβ for the treatment of eye
diseases involving ocular fibrosis, including

10   proliferative retinopathy (R.A. Pena et al. (ref.
below) , retinal detachment and post glaucoma (P.T.
Khaw et al., Eye 8 188-195, 1994) drainage surgery.
Connor et al. (J. Clin. Invest 83 1661-1666, 1989)
showed that much higher levels of TGFβ2 were present

15   in vitreous aspirates from patients with intraocular
fibrosis associated with proliferative retinopathy
compared with patients with uncomplicated retinal
detachment without ocular firbrosis and that the
biological activity of this TGFβ2 could be neutralised

20   with antibodies directed against TGFβ2. Moreover,
Pena et al. (Invest. Ophthalmology. Vis. Sci. 35:
2804-2808, 1994) showed that antibodies against TGFβ2
inhibit collagen contraction stimulated by TGFβ2.
Contraction of the vitreous gel by fibroblasts and

25   other cell types plays a critical role in the
proliferative retinopathy disease process, a process
thought to be mediated by TGFβ2.

There is other evidence pointing to TGFβ2 being

the most important TGFβ isoform promoting intraocular

fibrosis. TGFβ2 has been shown to be the predominant

isoform of TGFβ in the neural retina, retinal pigment

epithelium-choroid and vitreous of the human eye

5   (Pfeffer *et al. Exp. Eye Res.* 59: 323-333, 1994) and

found in human aqueous humour in specimens from eyes

undergoing cataract extraction with intraocular lens

implantation (Jampel *et al. Current Eye Research* 9:

963-969, 1990). Non-transformed human retinal pigment

10   epithelial cells predominantly secrete TGFβ2 (Kvanta

Opthalmic Res. 26: 361-367, 1994).

Other diseases which have potential for treatment

with antibodies against TGFβ include adult respiratory

distress syndrome, cirrhosis of the liver, post

15   myocardial infarction, post angioplasty restenosis,

keloid scars and scleroderma. The increase level of

expression of TGFβ2 in osteoporosis (Erlenbacher *et

al. J. Cell Biol.* 132: 195-210, 1996) means that htis

is a disease potentially treatable by antibodies

20   directed against TGFβ2.

The use of antibodies against TGFβ for the

treatment of diseases has been the subject of patent

applications for fibrotic disease (WO91/04748); dermal

scarring (WO92/17206); macrophage deficiency diseases

25   (PCT/US93/00998); macrophage pathogen infections

(PCT/US93/02017); neural scarring (PCT/US93/03068);

vascular disorders (PCT/US93/03795); prevention of

cataract (WO95/13827). The human antibodies against

human TGFβ disclosed in this application should be
valuable in these conditions.

It is shown herein that the human antibodies both
against human TGFβ1 and against human TGFβ2 can be
5 effective in the treatment of fibrosis in animal
models of neural scarring and glomerulonephritis in
either single chain Fv and whole antibody format.
This is the first disclosure of the effectiveness of
antibodies directed only against TGFβ2 as sole
10 treatment in these indications, although some
effectiveness of antibodies against TGFβ2 only has
been observed in a lung fibrosis model (Giri et al.
Thorax 48, 959-966, 1993 supra). The effectiveness of
the human antibodies against human TGFβ in treatment
15 of fibrotic disease has been determined by measuring a
decrease in the accumulation of components of the
extracellular matrix, including fibronectin and
laminin in animal models.

The evidence of efficacy of the antibodies
20 against TGFβ2 and TGFβ1 describe herein in prevention
of neural scarring in the animal model experiment
means that these antibodies are likely to be effective
in other disease states mediated by TGFβ. For
comparison, antisera isolated from turkeys directed
25 against TGFβ isoforms by Danielpour et al. (Cell
Physiol. 138: 79-86, 1989) have been shown to be
effective in the prevention of dermal scarring (Shah
et al. J. Cell Science 108: 985-1002, 1995), neural

scarring (Logan *et al.*, *supra*) and in *in vitro*

experiments relating to proliferative retinopathy

(Connor *et al.*, *supra*).

5   *TERMINOLOGY*

Specific binding member

This describes a member of a pair of molecules

which have binding specificity for one another. The

10   members of a specific binding pair may be naturally

derived or wholly or partially synthetically produced.

One member of the pair of molecules has an area on its

surface, or a cavity, which specifically binds to and

is therefore complementary to a particular spatial and

15   polar organisation of the other member of the pair of

molecules. Thus the members of the pair have the

property of binding specifically to each other.

Examples of types of specific binding pairs are

antigen-antibody, biotin-avidin, hormone-hormone

20   receptor, receptor-ligand, enzyme-substrate. This

application is concerned with antigen-antibody type

reactions.

Antibody

25   This describes an immunoglobulin whether natural

or partly or wholly synthetically produced. The term

also covers any polypeptide or protein having a

binding domain which is, or is homologous to, an

antibody binding domain. These can be derived from natural sources, or they may be partly or wholly synthetically produced. Examples of antibodies are the immunoglobulin isotypes and their isotypic

5   subclasses; fragments which comprise an antigen binding domain such as Fab, scFv, Fv, dAb, Fd; and diabodies.

It is possible to take monoclonal and other antibodies and use techniques of recombinant DNA

10   technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an

15   antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB 2188638A or EP-A-239400. A hybridoma or other cell producing an antibody may be subject to genetic mutation or other

20   changes, which may or may not alter the binding specificity of antibodies produced.

As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding member or substance

25   having a binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an

immunoglobulin binding domain, whether natural or
wholly or partially synthetic. Chimeric molecules
comprising an immunoglobulin binding domain, or
equivalent, fused to another polypeptide are therefore
5   included. Cloning and expression of chimeric
antibodies are described in EP-A-0120694 and EP-A-
0125023.

It has been shown that fragments of a whole
antibody can perform the function of binding antigens.
10   Examples of binding fragments are (i) the Fab fragment
consisting of VL, VH, CL and CH1 domains; (ii) the Fd
fragment consisting of the VH and CH1 domains; (iii)
the Fv fragment consisting of the VL and VH domains of
a single antibody; (iv) the dAb fragment (Ward, E.S.
15   et al., Nature 341, 544-546 (1989)) which consists of
a VH domain; (v) isolated CDR regions; (vi) F(ab')2
fragments, a bivalent fragment comprising two linked
Fab fragments (vii) single chain Fv molecules (scFv),
wherein a VH domain and a VL domain are linked by a
20   peptide linker which allows the two domains to
associate to form an antigen binding site (Bird et al,
Science, 242, 423-426, 1988; Huston et al, PNAS USA,
85, 5879-5883, 1988); (viii) bispecific single chain
Fv dimers (PCT/US92/09965) and (ix) "diabodies",
25   multivalent or multispecific fragments constructed by
gene fusion (WO94/13804; P. Holliger et al Proc. Natl.
Acad. Sci. USA 90 6444-6448, 1993).

Diabodies are multimers of polypeptides, each

polypeptide comprising a first domain comprising a
binding region of an immunoglobulin light chain and a
second domain comprising a binding region of an
immunoglobulin heavy chain, the two domains being
5   linked (e.g. by a peptide linker) but unable to
associate with each other to form an antigen binding
site: antigen binding sites are formed by the
association of the first domain of one polypeptide
within the multimer with the second domain of another
10   polypeptide within the multimer (WO94/13804).

     Where bispecific antibodies are to be used, these
may be conventional bispecific antibodies, which can
be manufactured in a variety of ways (Holliger, P. and
Winter G. Current Opinion Biotechnol. 4, 446-449
15   (1993)), eg prepared chemically or from hybrid
hybridomas, or may be any of the bispecific antibody
fragments mentioned above. It may be preferable to
use scFv dimers or diabodies rather than whole
antibodies. Diabodies and scFv can be constructed
20   without an Fc region, using only variable domains,
potentially reducing the effects of anti-idiotypic
reaction. Other forms of bispecific antibodies
include the single chain "Janusins" described in
Traunecker et al, Embo Journal, 10, 3655-3659, (1991).
25   Bispecific diabodies, as opposed to bispecific
whole antibodies, may also be particularly useful
because they can be readily constructed and expressed
in E.coli. Diabodies (and many other polypeptides

such as antibody fragments) of appropriate binding specificities can be readily selected using phage display (WO94/13804) from libraries. If one arm of the diabody is to be kept constant, for instance, with

5   a specificity directed against antigen X, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected.

Antigen binding domain

10   This describes the part of an antibody which comprises the area which specifically binds to and is complementary to part or all of an antigen. Where an antigen is large, an antibody may only bind to a particular part of the antigen, which part is termed

15   an epitope. An antigen binding domain may be provided by one or more antibody variable domains. Preferably, an antigen binding domain comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

20

Specific

This may be used to refer to the situation in which one member of a specific binding pair will not show any significant binding to molecules other than

25   its specific binding partner(s). The term is also applicable where e.g. an antigen binding domain is specific for a particular epitope which is carried by a number of antigens, in which case the specific

binding member carrying the antigen binding domain
will be able to bind to the various antigens carrying
the epitope.

5   *Neutralisation*

This refers to the situation in which the binding
of a molecule to another molecule results in the
abrogation or inhibition of the biological effector
function of the another molecule.

10

Functionally equivalent variant form

This refers to a molecule (the variant) which
although having structural differences to another
molecule (the parent) retains some significant

15   homology and also at least some of the biological
function of the parent molecule, e.g. the ability to
bind a particular antigen or epitope. Variants may be
in the form of fragments, derivatives or mutants. A
variant, derivative or mutant may be obtained by

20   modification of the parent molecule by the addition,
deletion, substitution or insertion of one or more
amino acids, or by the linkage of another molecule.
These changes may be made at the nucleotide or protein
level. For example, the encoded polypeptide may be a

25   Fab fragment which is then linked to an Fc tail from
another source. Alternatively, a marker such as an
enzyme, flourescein, etc, may be linked.

Comprise

This is generally used in the sense of include,
that is to say permitting the presence of one or more
features or components.

5

The present invention generally provides a
specific binding member comprising an antibody antigen
binding domain. More particularly it provides a
specific binding member for TGFβ, particularly the

10   isoforms TGFβ2, TGFβ1, or TGFβ1 and TGFβ2.

The present invention provides a specific binding
member which comprises a human antibody antigen
binding domain specific for TGFβ1 and/or TGFβ2 and
which has low cross reactivity with TGFβ3. The cross-

15   reactivity may be as assessed using any or all of the
following assays: biosensor (e.g. BIACore™), ELISA and
radioreceptor. The present invention provides
specific binding member which comprises a human
antibody antigen binding domain specific for TGFβ1

20   and/or TGFβ2 which binds preferentially to these
isoforms compared with TGFβ3.

The TGFβ may be human TGFβ.

The specific binding member may be in the form of
an antibody fragment such as single chain Fv (scFv).

25   Other types of antibody fragments may also be utilised
such as Fab, Fab', F(ab')$_2$, Fabc, Facb or a diabody
(G.Winter & C.Milstein Nature 349, 293-299, 1991;
WO94/13804). The specific binding member may be in

the form of a whole antibody. The whole antibody may

be in any of the forms of the antibody isotypes eg

IgG, IgA, IgE, and IgM and any of the forms of the

isotype subclasses eg IgG1 or IgG4.

5   The specific binding member may also be in the

form of an engineered antibody eg bispecific antibody

molecules (or fragments such as $F(ab')_2$) which have

one antigen binding arm (ie specific binding domain)

against TGFβ and another arm against a different

10   specificity. Indeed the specific binding members

directed against TGFβ1 and/or TGFβ2 described herein

may be combined in a bispecific diabody format. For

example the antibodies 31G9 directed against TGFβ1 and

6H1 directed against TGFβ2 may be combined to give a

15   single dimeric molecule with both specificities.

The binding domain may comprise part or all of a

VH domain encoded by a germ line gene segment or a re-

arranged gene segment. The binding domain may

comprise part or all of either a VL kappa domain or a

20   VL lambda domain.

The binding domain may be encoded by an altered

or variant form of a germ line gene with one or more

nucleotide alterations (addition, deletion,

substitution and/or insertion), e.g. about or less

25   than about 25, 20, 15, 10 or 5 alterations, 4, 3, 2 or

1, which may be in one or more frameworks and/or

CDR's.

The binding domain may comprise a VH3 gene

sequence of one of the following germ lines; the DP49

germ line; the DP53 germ line; the DP50 germ line; the

DP46 germ line; or a re-arranged form thereof.

5   A preferred VH domain for anti-TGFβ2 specific

binding members according to the present invention is

that of 6H1 VH, whose sequence is shown in Figure 2(a)

(i).   6H1 may be paired with a variety of VL domains,

as exemplified herein. Amino acid sequence variants

of 6H1 VH may be employed.

10   The specific binding member may neutralise the *in

vitro* and/or *in vivo* effect of TGFβ, that is one or

more of the isoforms, particularly TGFβ1 and/or TGFβ2.

The specific binding member may be a high

affinity antibody. Preferred affinities are discussed

15   elsewhere herein.

The binding domain may comprise part or all of a

VH domain having either an amino acid sequence as

shown in Fig 1(a)(i) or (ii) or Fig 1(c)(i) or a

functionally equivalent variant form of a said amino

20   acid sequence.

The binding domain may comprise part or all of a

VH domain encoded by either a nucleotide sequence as

shown in Fig 1(a)(i) or (ii) or Fig 1(c)(i) or a

functionally equivalent variant form of a said

25   nucleotide sequence.

The binding domain may comprise part or all of a

VL domain having either an amino acid sequence as

shown in Fig 1(a)(iii) or Fig 1(b) or a functionally

equivalent variant form of a said amino acid sequence.

The binding domain may comprise part or all of a
VL domain encoded by either a nucleotide sequence as
shown in Fig 1(a)(iii) or Fig 1(b) or a functionally

5   equivalent variant form of a said nucleotide sequence.

The binding domain may comprise part or all of a
VH domain having a variant form of the Fig 1(a)(i)
amino acid, the variant form being one of those as
provided by Fig 3.

10   The binding domain may comprise part or all of a
VH domain having either an amino acid sequence as
shown in Fig 2(a)(i) or (ii) or a functionally
equivalent variant form of a said amino acid sequence.

The binding domain may comprise part or all of a

15   VH domain encoded by either a nucleotide sequence as
shown in Fig 2(a)(i) or (ii) or a functionally
equivalent variant form of a said nucleotide sequence.

The binding domain may comprise part or all of a
VL domain having either an amino acid sequence as

20   shown in any of Figs 2(b)(i) to (v) or a functionally
equivalent variant form of a said amino acid sequence.

The binding domain may comprise part or all of a
VL domain encoded by either a nucleotide sequence as
shown in any of Figs 2(b)(i) to (v) or a functionally

25   equivalent variant form of a said nucleotide sequence.

The binding domain may be specific for both TGFβ1
and TGFβ2. The binding domain may be specific for
both human TGFβ1 and human TGFβ2. The specific

binding member may be in the form of scFv.

The binding domain may comprise part or all of a VL domain having either an amino acid sequence as shown in Fig 4 or a functionally equivalent variant

5   form of said amino acid sequence. The binding domain may comprise part or all of a VL domain encoded by either the nucleotide sequence as shown in Fig 4 or a functionally equivalent variant form of said nucleotide sequence.

10   In particular, the binding domain may comprise one or more CDR (complementarity determining region) with an amino acid sequence shown in any of the figures. In a preferred embodiment, the binding domain comprises one or more of the CDRs, CDR1, CDR2

15   and/or CDR3 shown in the Figures, especially any of those shown in Figure 19. In a preferred embodiment, the binding domain comprises a VH CDR3 sequence as shown, especially as shown in Figure 19. Functionally equivalent variant forms of the CDRs are encompassed

20   by the present invention, in particular variants which differ from the CDR sequences shown by addition, deletion, substitution or insertion of one or more amino acids and which retain ability to bind the antigen and optionally one or more of the preferred

25   characteristics for specific binding members of the present invention as disclosed herein. The specific binding member may comprise all or part of the framework regions shown flanking and between the CDRs

in the Figures, especially Figure 19, or different

framework regions including modified versions of those

shown.

So-called "CDR-grafting" in which one or more CDR

5   sequences of a first antibody is placed within a

framework of sequences not of that antibody, e.g. of

another antibody is disclosed in EP-B-0239400.

The present invention also provides a polypeptide

with a binding domain specific for TGFβ which

10   polypeptide comprises a substantial part or all of

either an amino acid sequence as shown in any of Fig

1(a), Fig 1(b), Fig 1(c), Fig 2(a), Fig 2(b), Fig 4

or a functionally equivalent variant form of a said

amino acid sequence. The polypeptide may comprise a

15   substantial part or all of an amino acid sequence

which is a functionally equivalent variant form of the

Fig 1(a)(i) amino acid sequence, the variant being one

of those variants as shown in Fig 3.

Variable domain amino acid sequence variants of

20   any of the VH and VL domains whose sequences are

specifically disclosed herein may be employed in

accordance with the present invention, as discussed.

Particular variants may include one or more amino acid

sequence alterations (addition, deletion, substitution

25   and/or insertion), maybe less than about 20

alterations, less than about 15 alterations, less than

about 10 alterations or less than about 5 alterations,

4, 3, 2 or 1. Alterations may be made in one or more

framework regions and/or one or more CDR's.

A specific binding member according to the invention may be one which competes for binding to TGFβ1 and/or TGFβ2 with any specific binding member
5   which both binds TGFβ1 and/or TGFβ2 and comprises part of all of any of the sequences shown in the Figures. Competition between binding members may be assayed easily *in vitro*, for example by tagging a specific reporter molecule to one binding member which can be
10   detected in the presence of other untagged binding member(s), to enable identification of specific binding members which bind the same epitope or an overlapping epitope.

Preferred specific binding members for TGFβ1
15   compete for binding to TGFβ1 with the antibody CS37, discussed in more details elsewhere herein.

Preferred specific binding members for TGFβ2 compete for binding to TGFβ2 with the antibody 6B1 discussed in more detail elsewhere herein. They may
20   bind the epitope RVLSL or a peptide comprising the amino acid sequence RVLSL, particularly such a peptide which adopts an α-helical conformation. They may bind the peptide TQHSRVLSLYNTIN. In testing for this, a peptide with this sequence plus CGG at the N-terminus
25   may be used. Specific binding members according to the present invention may be such that their binding for TGFβ2 is inhibited by a peptide comprising RVLSL, such as a peptide with the sequence TQHSRVLSLYNTIN.

In testing for this, a peptide with this sequence plus CGG at the N-terminus may be used.

TQHSRVLSLYNTIN corresponds to the alpha helix H3 (residues 56-69) of TGFβ2, as discussed elsewhere herein. The equivalent region in TGFβ1 has the sequence TQYSKVLSLYNQHN. Anti-TGFβ1 antibodies which bind this region are of particular interest in the present invention, and are obtainable for example by panning a peptide with this sequence (or with CGG at the N-terminus) against a phage display library. Specific binding members which bind the peptide may be selected by means of their binding, and may be neutralising for TGFβ1 activity. Binding of such specific binding members to TGFβ1 may be inhibited by the peptide TQYSKVLSLYNQHN (optionally with CGG at the N-terminus).

A specific binding member according to the present invention which is specific for TGFβ2 may show no or substantially no binding for the latent form of TGFβ2, i.e. be specific for the active form of TGFβ2. 6B1 is shown in Example 6 to have this property.

6B1 is particularly suitable for therapeutic use in the treatment of fibrotic disorders because it has the following advantageous properties. 6B1 binds to TGFβ2 with a dissociation constant of 2.3nM in the single chain form and 0.89nM for the whole antibody form, 6B1 IgG4 (Example 13). The antibody 6B1 IgG4 neutralises the biological activity of TGFβ2 in an

antiproliferation assay (IC_{50} 2nM; examples 7 and 10) and in a radioreceptor assay (IC_{50} less than 1nM; Table 6). The antibody binds to the peptide TQHSRVLSLYNTIN ($TGF\beta2_{56-69}$) from the alpha helix H3 of $TGF\beta2$ and recognises the corresponding peptide from $TGF\beta1$ more weakly. 6B1 recognises the active but not the latent form of $TGF\beta2$ (Example 6), recognises $TGF\beta2$ in mammalian tissues by ICC and does not bind non-specifically to other human tissues (Example 12). The antibody preferentially binds to $TGF\beta2$ as compared to $TGF\beta3$, the cross-reactivity with $TGF\beta3$ being 9% as determined by the ratio of the dissociation constants.

The other antibodies described in this application which contain the 6H1 VH domain, 6H1 and 6A5 have similar properties. The dissociation constants of were determined to be 2nM for 6B1 IgG4 (Example 2) and 0.7nM for 6A5 single chain Fv (Table 1). 6H1 IgG4 neutralises the biological activity of $TGF\beta2$ with IC_{50} values of 12 to 15nM (Examples 7 and 10). 6A5 and 6H1 inhibit receptor binding of $TGF\beta2$ in a radioreceptor assay with IC_{50} values of about 1nM in the single chain Fv format and 10nM or below in the whole antibody, IgG4 format. Both 6H1 IgG4 and 6A5 scFv were shown to be effective in the prevention of neural scarring (Example 5).

Therefore for the first human antibodies directed against $TGF\beta2$ are provided which have suitable properties for treatment of diseases characterised by

32

the deleterious presence of TGFβ2.   Such antibodies

preferably neutralise TGFβ2 and preferably have a

dissociation constant for TGFβ2 of less than about

100nM, more preferably about 10nM, more preferably

5   below about 5nM.   The antibodies preferentially bind

to TGFβ2 as compared to TGFβ3, preferably have less

than 20% cross-reactivity with TGFβ3 (as measured by

the ratio of the dissociation constants) and

preferably have less than about 10% cross-reactivity.

10   The antibody preferably recognises the active but not

the latent form of TGFβ2.

For antibodies against TGFβ1, the properties

desired for an antibody to be effective in treatment

of fibrotic disease are similar.   Such antibodies

15   preferably neutralise TGFβ1 and have a dissociation

constant for TGFβ1 of less than about 100nM, more

preferably below about 10nM, more preferably below

about 5nM.   The antibodies preferentially bind to

TGFβ1 as compared to TGFβ3, preferably have less than

20   about 20% cross-reactivity with TGFβ3 (as measured by

the ratio of the dissociation constants) and more

preferably have less than about 10% cross-reactivity.

The antibody preferably recognises the active but not

the latent form of TGFβ1.   The antibody 31G9 has a

25   dissociation constant of 12nM (Table 5).   The

antibodies CS37 scFv and 27C1/10A6 IgG4 show IC_{50}

values in a radioreceptor assay of 8nM and 9nM

respetively, indicating a dissociation contstant in

the low nanomolar range. 27C1/10A6 IgG4 was shown to
be effective in a neural scarring model. Cross-
reactivity of antibodies of the 1B2 lineage with TGFβ3
is very low (Example 9).

5   In addition to an antibody sequence, the specific
binding member may comprise other amino acids, e.g.
forming a peptide or polypeptide, or to impart to the
molecule another functional characteristic in addition
to ability to bind antigen. For example, the specific

10   binding member may comprise a label, an enzyme or a
fragment thereof and so on.

The present invention also provides a
polynucleotide which codes for a polypeptide with a
binding domain specific for TGFβ which polynucleotide

15   comprises a substantial part or all of a nucleotide
sequence which codes for either an amino acid sequence
as shown in any one of Fig 1(a), Fig 1(b), Fig 1(c),
Fig 2(a), Fig 2(b), Fig 4 or a functionally equivalent
variant form of a said amino acid sequence. The

20   polynucleotide may code for a polypeptide with a
binding domain specific for TGFβ which polynucleotide
comprises a substantial part or all of a nucleotide
sequence which codes for an amino acid sequence which
is a functionally equivalent variant form of the Fig

25   1(a)(i) amino acid sequence, the variant being one of
those as shown in Fig 3. The polynucleotide may code
for a polypeptide with a binding domain specific for
TGFβ which polynucleotide comprises a substantial

part or all of a either a nucleotide sequence as shown
in any of Fig 1(a), Fig 1(b), Fig 1(c), Fig 2(a), Fig
2(b), Fig 4 or a functionally equivalent variant form
of said nucleotide sequence. The polynucleotide may

5   code for a polypeptide with a binding domain specific
for TGFβ which polynucleotide comprises a substantial
part or all a nucleotide sequence which codes for a
variant form of the Fig 1(a)(i) amino acid sequence,
the variant being one of those as shown in Fig 3.

10   The present invention also provides constructs in
the form of plasmids, vectors, transcription or
expression cassettes which comprise least one
polynucleotide as above.

The present invention also provides a recombinant

15   host cell which comprises one or more constructs as
above.

A specific binding member according to the
present invention may be made by expression from
encoding nucleic acid. Nucleic acid encoding any

20   specific binding member as provided itself forms an
aspect of the present invention, as does a method of
production of the specific binding member which method
comprises expression from encoding nucleic acid
therefor. Expression may conveniently be achieved by

25   culturing under appropriate conditions recombinant
host cells containing the nucleic acid. Following
production by expression a specific binding member may
be isolated and/or purified using any suitable

technique, then used as appropriate.

Specific binding members and encoding nucleic acid molecules and vectors according to the present invention may be provided isolated and/or purified,

5   e.g. from their natural environment, in substantially pure or homogeneous form, or, in the case of nucleic acid, free or substantially free of nucleic acid or genes origin other than the sequence encoding a polypeptide with the required function. Nucleic acid

10   according to the present invention may comprise DNA or RNA and may be wholly or partially synthetic. The term "isolate" encompasses all these possibilities.

The nucleic acid may encode any of the amino acid sequences shown in any of the Figures, or any

15   functionally equivalent form. The nucleotide sequences employed may be any of those shown in any of the Figures, or may be a variant, allele or derivative thereof. Changes may be made at the nucleotide level by addition, substitution, deletion or insertion of

20   one or more nucleotides, which changes may or may not be reflected at the amino acid level, dependent on the degeneracy of the genetic code.

Systems for cloning and expression of a polypeptide in a variety of different host cells are

25   well known. Suitable host cells include bacteria, mammalian cells, yeast and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include

Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells and many others. A common, preferred bacterial host is *E. coli*.

5   The expression of antibodies and antibody fragments in prokaryotic cells such as *E. coli* is well established in the art. For a review, see for example Plückthun, A. Bio/Technology 9: 545-551 (1991). Expression in eukaryotic cells in culture is also available to those skilled in the art as an option for

10   production of a specific binding member, see for recent reviews, for example Reff, M.E. (1993) Curr. Opinion Biotech. 4: 573-576; Trill J.J. et al. (1995) Curr. Opinion Biotech 6: 553-560.

Suitable vectors can be chosen or constructed,

15   containing appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as

20   appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in

25   preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second

Edition, Ausubel et al. eds., John Wiley & Sons, 1992.
The disclosures of Sambrook et al. and Ausubel et al.
are incorporated herein by reference.

5   Thus, a further aspect of the present invention
provides a host cell containing nucleic acid as
disclosed herein. A still further aspect provides a
method comprising introducing such nucleic acid into a
host cell. The introduction may employ any available
technique. For eukaryotic cells, suitable techniques
10   may include calcium phosphate transfection, DEAE-
Dextran, electroporation, liposome-mediated
transfection and transduction using retrovirus or
other virus, e.g. vaccinia or, for insect cells,
baculovirus. For bacterial cells, suitable techniques
15   may include calcium chloride transformation,
electroporation and transfection using bacteriophage.

The introduction may be followed by causing or
allowing expression from the nucleic acid, e.g. by
culturing host cells under conditions for expression
20   of the gene.

In one embodiment, the nucleic acid of the
invention is integrated into the genome (e.g.
chromosome) of the host cell. Integration may be
promoted by inclusion of sequences which promote
25   recombination with the genome, in accordance with
standard techniques.

The present invention also provides a method
which comprises using a construct as stated above in

an expression system in order to express a specific binding member or polypeptide as above.

Following production of a specific binding member it may be used for example in any of the manners disclosed herein, such as in the formulation of a composition, pharmaceutical or a diagnostic product, such as a kit comprising in addition to the specific binding member one or more reagents for determining binding of the member to cells, as discussed. A composition may comprise at least one component in addition to the specific binding member.

The present invention also provides pharmaceuticals which comprise a specific binding member as above, optionally with one or more excipients.

The present invention also provides the use of a specific binding member as above in the preparation of a medicament to treat a condition in which it is advantageous to counteract the fibrosis promoting effects of TGFβ. The condition may be a fibrotic condition characterized by an accumulation in a tissue of components of the extracellular matrix. The components of the extracellular matrix may be fibronectin or laminin.

The condition may be selected from the group consisting of: glomerulonephritis, neural scarring, dermal scarring, ocular scarring, lung fibrosis, arterial injury, proliferative retinopathy, retinal

detachment, adult respiratory distress syndrome, liver

cirrhosis, post myocardial infarction, post

angioplasty restenosis, keloid scarring, scleroderma,

vascular disorders, cataract, glaucoma, proliferative

5   retinopathy.

The condition may be neural scarring or

glomerulonephritis.

The present invention also provides the use of a

specific binding member as above, in the preparation

10   of a medicament to treat an immune/inflammatory

disease condition in which it is advantageous to

counteract the effects of TGFβ. Illustrative

conditions are rheumatoid arthritis, macrophage

deficiency disease and macrophage pathogen infection.

15   The present invention also provides a method

which comprises administering to a patient a

therapeutically effective amount of a specific binding

member as above in order to treat a condition in which

it is advantageous to counteract the fibrosis

20   promoting effects of TGFβ. Fibrotic conditions are

listed above.

The present invention also provides a method

which comprises administering to a patient a

prophylactically effective amount of a specific

25   binding member as above in order to prevent a

condition in which it is advantageous to prevent the

fibrosis promoting effects of TGFβ. Fibrotic

conditions are listed above.

The present invention also provides methods which comprise administering to patients prophylactically and/or therapeutically effective amounts of a specific binding member as above in order to prevent or treat

5 an immune/inflammatory disease condition in which it is advantageous to counteract the effects of TGFβ. Illustrative conditions are stated above.

Thus, various aspects of the invention provide methods of treatment comprising administration of a

10 specific binding member as provided, pharmaceutical compositions comprising such a specific binding member, and use of such a specific binding member in the manufacture of a medicament for administration, for example in a method of making a medicament or

15 pharmaceutical composition comprising formulating the specific binding member with a pharmaceutically acceptable excipient.

In accordance with the present invention, compositions provided may be administered to

20 individuals, which may be any mammal, particularly rodent, e.g. mouse, horse, pig, sheep, goat, cattle, dog, cat or human. Administration is preferably in a "therapeutically effective amount", this being sufficient to show benefit to a patient. Such benefit

25 may be at least amelioration of at least one symptom. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription

of treatment, eg decisions on dosage etc, is within the responsibility of general practioners and other medical doctors. Appropriate doses of antibody are well known in the art; see Ledermann J.A. et al.

5 (1991) Int J. Cancer 47: 659-664; Bagshawe K.D. et al. (1991) Antibody, Immunoconjugates and Radiopharmaceuticals 4: 915-922.

A composition may be administered alone or in combination with other treatments, either

10 simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may comprise, in addition to active

15 ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature

20 of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or

25 liquid form. A tablet may comprise a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral

oil or synthetic oil. Physiological saline solution,
dextrose or other saccharide solution or glycols such
as ethylene glycol, propylene glycol or polyethylene
glycol may be included.

5   For intravenous, injection, or injection at the
site of affliction, the active ingredient will be in
the form of a parenterally acceptable aqueous solution
which is pyrogen-free and has suitable pH, isotonicity
and stability. Those of relevant skill in the art are
10   well able to prepare suitable solutions using, for
example, isotonic vehicles such as Sodium Chloride
Injection, Ringer's Injection, Lactated Ringer's
Injection. Preservatives, stabilisers, buffers,
antioxidants and/or other additives may be included,
15   as required.

       Further aspects of the invention and embodiments
will be apparent to those skilled in the art. In
order that the present invention is fully understood,
20   the following examples are provided by way of
exemplification only and not by way of limitation.
       Reference is made to the following figures.

       Figure 1 shows the DNA and protein sequences of
25   antibodies specific for TGFβ1. Figure 1(a) shows the
amino acid and encoding nucleic acid sequences of
antibody variable domains of antibodes to TGFβ1
isolated directly from repertoires: Figure 1(a)(i)-

1B2 VH (also known as 7A3 VH); Figure 1(a) (ii) - 31G9

VH; Figure 1(a) (iii) - 31G9 VL. Figure 1 (b) shows

the amino acid and encoding nucleic acid sequences of

antibody light chain variable domains of antibodies to

5   TGFβ1 isolated by chain shuffling: Figure 1(b) (i) -

7A3 VL; Figure 1(b) (ii) - 10A6 VL. Figure 1(c) (i)

shows the amino acid and encoding nucleic acid

sequences for 27C1 VH, from an antibody to TGFβ1

isolated from a CDR3 spiking experiment.

10   Figure 2 shows the DNA and protein sequences of

antibodies specific for TGFβ2. Figure 2(a) shows

amino acid and encoding nucleic acid sequences for

variable domains of antibodies to TGFβ2 isolated

directly from repertoires: Figure 2(a) (i) - 2A-H11 VH

15   (also known as 6H1 VH); Figure 2(a) (ii) - 2A-A9 VH

(also known as 11E6 VH). Figure 2(b) shows amino acid

and encoding nucleic acid sequences of antibody

variable domains of antibodies specific for TGFβ2

isolated following chain shuffling: Figure 2(b) (i) -

20   6H1 VL; Figure 2(b) (ii) - 6A5 VL; Figure 2(b) (iii) -

6B1 VL; Figure 2(b) (iv) 11E6 VL; (v) Figure 2(b) (v)

- 14F12 VL.

         Figure 3 shows the protein sequences of VH CDR3

of clones derived from 1B2 by 'spiking' mutagenesis.

25   Differences from 1B2 VH CDR3 are in bold.

         Figure 4 shows the DNA and protein sequence of

the VH and VL domains of VT37, cross-reactive between

TGFβ1 and TGFβ2.

44

Figure 5 shows the DNA sequence and encoded amino acid sequence in the region of the heavy chain VH leader from the vector vhcassette2. Restriction enzymes HindIII, SfiI, PstI, BstEII, BamHI and EcoRI

5   cut at the points indicated.

Figure 6 shows a map of the vector pG4D100 (not to scale). Multiple cloning site (MCS): 5'-HindIII-PacI-BamHI-(XanI)-(PmlI)-(NheI)-AscI-(BssHII)-XhoI-PmeI-BsiWI-3'. Restriction sites shown in brackets

10   are not unique.

Figure 7 shows the DNA sequence, including intron, and encoded amino acid sequence in the region of the light chain VL leader for the vector vlcassette1 (vlcassette CAT1). Restrcition enzymes

15   HindIII, ApaLI, SacI, XhoI and BamHI cut at the sites indicated (ApaLI within the leader).

Figure 8 shows a map of the vector pLN10 (not to scale). Multiple cloning site (MCS): 5'-HindIII-(SphI)-(PstI)-SalI-XbaI-BamHI-3' (1224-1259.

20   Restriction sites shown in brackets are not unique.

Figure 9 shows a map of the vector pKN100 (not to scale). Multiple cloning site (MCS): 5'-MluI-(AvaI)-HindIII-(SphI)-(PstI)-SalI-XbaI-BamHI-3'. Restriction sites shown in brackets are not unique.

25   Figure 10 shows the % neutralisation of TGFβ2 activity by single chain Fv antibodies in an assay using proliferation of the erythroleukaemia cell line TF1 at different nM concentrations of scFv.

Figure 11 shows the neutralisation of TGFβ2
activity by whole IgG4 antibodies in an assay using
proliferation of the erythroleukaemia cell line TF1 at
different nM concentrations of antibody.

5   Figure 12 shows the effect of treatment of
animals with antibodies on neural scarring as measured
by the deposition of (Figure 12(a)) fibronectin and
(Figure 12(b)) laminin detected using integrated
fluorescence intensity. The graphs show scatter plots
10 of individual animal data points. The bar graph shows
the mean integrated fluorescence intensity of the
group.

Figure 13 shows the results of an ELISA to
measure the cross-reactivity of the antibodies 6B1
15 IgG4 and 6A5 IgG4 with TGFβ isoforms and non-specific
antigens. Figure 13(a) shows cross-reactivty of 6B1
IgG4 to a panel of non-specific antigens and TGFβ's,
plotting OD405nm for each antigen: 1 - interleukin 1;
2 - human lymphotoxin (TNFβ); 3 - human insulin; 4 -
20 human serum albumin; 5 - ssDNA; 6 - oxazolone-bovine
serum albumin; 7 - keyhole limpet haemocyanin; 8 -
chicken egg white trypsin inhibitor; 9 -
chymotrypsinogen; 10 - cytochrome C; 11 - GADPH; 12 -
ovalbumin; 13 - hen egg lysozyme; 14 - bovine serum
25 albumin; 15 - TNFα; 16 - TGFβ1; 17 - TGFβ2; 18 -
TGFβ3; 19 - PBS only. Figure 13(b) shows the OD405nm
for the antibody 6A5 IgG4 against the same panel of
antigens. For both Figure 13(a) and Figure 13(b),

antigens 1 to 15 were used for coating the plate at a
concentration of 10µg/ml in PBS. The TGFbetas were
coated at 0.2µg/ml in PBS. Coating was performed at
4°C overnight. 100µg of each antigen was used per
5   well and duplicates of each antigen for each IgG to be
tested. IgG samples were incubated with the coated
antigens at 37°C for 2 hours after blocking with 2%
marvel-PBS. The labelled second antibody was a mouse
anti-human Fc1 alkaline phosphatase conjugated and the
10   substrate used to detect bound second antibody was
PNPP at 1mg/ml with the absorbance read at 405nm.

Figure 14 shows the amino acid and encoding
nucleic acid sequence for the VL domain of the TGFβ1-
specific antibody CS37.

15   Figure 15 shows data from an ELISA detecting
binding of 6B1 IgG4 to BSA conjugated with either
peptide TGFβ2$_{56-69}$ or peptide TGFβ1$_{56-69}$ coated on to an
ELISA plate. 6B1 IgG4 was incubated at various
concentrations in µg/ml and the absorbance at 405nm
20   measured after addition of the detection agents.
OD405nm results are plotted at the various
concentrations for BSA-TGFβ2$_{56-69}$ ("Beta2 peptide" -
diamonds) and BSA-TGFβ1$_{56-69}$ ("Beta1 peptide" -
squares).

25   Figure 16 shows % neutralisation of TGF-β2 anti-
proliferative effect on TF1 cells by whole antibodies,
6H1 IgG4, 6B1 IgG4 and the mouse monoclonal from
Genzyme, at various concentrations (nM IgG).

Figure 17 shows % neutralisation of TGF-β1 anti-proliferative effect on TF1 cells by whole antibodies,6H1 IgG4, 6B1 IgG4 and the mouse monoclonal from Genzyme, at various concentrations (nM IgG).

5   Figure 18 shows % neutralisation of TGF-β3 anti-proliferative effect on TF1 cells by whole antibodies, 6H1 IgG4, 6B1 IgG4 and the mouse monoclonal from Genzyme, at various concentrations (nM IgG).

Figure 19 shows amino acid and encoding DNA

10   sequences of regions of antibodies directed against TGFβ2 showing CDR sequences in italics: Figure 19(i) 2A-H11 VH (also known as 6H1 VH); Figure 19(ii) 6B1 VL; Figure 19(iii) 6A5 VL and Figure 19(iv) 6H1 VL.

Figure 20 shows the vector p6H1 VH-gamma4 (7263

15   bp). The gene encoding 6H1 VH is inserted as a HindIII-ApaI restriction fragment.

Figure 21 shows the vector p6B1 lambda (10151 bp). The gene encoding 6B1 VL is inserted as an EcoRI-BstBI restriction fragment.

20   Figure 22 shows the vector p6B1 gamma4gs (14176 bp). The genes encoding the heavy and light chains of 6BI IgG4 are combined in a single vector.

Figure 23 shows the results of competition ELISA experiments described in Example 6. Following

25   overnight incubation with TGFβ2, plates were treated with the following solutions 1-4 (number corresponding to those in Figure): 1 - 400μl Hams F12/DMEM (reagent blank), 2 - 400μl Hams F12/DMEM plus 4μg 6B1 IgG4

antibody (positive control), 3 - 400µl PC3 untreated

conditioned media plus 4µg 6B1 IgG4 antibody (latent

TGFβ$_2$ sample), 4 - 400µl PC3 acid activated

conditioned media plus 4µg 6B1 IgG4 antibody (active

5   TGFβ$_2$ sample).

All documents mentioned herein are incorporated

by reference.

10   **List of Examples**

Example 1 - Isolation of antibodies specific for

TGFβ1, antibodies specific for TGFβ2 and antibodies

specific for TGFβ1 and TGFβ2.

15   Example 2 - Construction of cell lines expressing

whole antibodies.

Example 3 - Neutralisation of TGFβ activity by

antibodies assessed using in vitro assays.

Example 4 - Inhibition by antibodies of TGFβ

20   binding to receptors.

Example 5 - Prevention of neural scarring using

antibodies against TGFβ.

Example 6 - Determination of Binding of 6B1 IgG4

to Active or Latent Form of TGFβ$_2$.

25   Example 7 - Neutralisation by antibodies directed

against TGFβ2 of the inhibitory effect of TGFβ

isoforms on cells proliferation.

Example 8 - Inhibition by antibodies directed

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against TGFβ2 of binding of other TGFβ isoforms to
receptors measured in a radioreceptor assay.

Example 9 - Assessment of TGFβ1 antibodies for
potential therapeutic use.

5   Example 10 - Construction of a high expressing
cell line for 6B1 IgG4 using the glutamine synthase
selection system and assessment in a neutralisation
assay.

Example 11 - Determination of the epitope on

10   TGFβ2 for the antibody 6B1 using a peptide phage
display library.

Example 12 - Determination of the binding of 6B1
IgG4 to tissues by immunocytochemistry (ICC).

Example 13 - Determination of the kinetic

15   parameters of 6B1 IgG4 and single chain Fv for binding
to TGFβ2.

Example 14 - Binding of a Peptide Corresponding
to Residues 56 to 69 of TGFβ2 to 6B1 IgG4.

20   *EXAMPLE 1*

*Isolation and Characterisation of Antibodies Binding
to TGFβ1 and TGFβ2*

1 Identification and Characterisation of Antibodies to

25   Human TGFb-1 by Selection of Naive and Synthetic Phage
Antibody Repertoires

Antibody repertoires

The following antibody repertoires were used:

1. <u>Peripheral blood lymphocyte (PBL) library</u> derived

from unimmunized human (Marks, J. D., Hoogenboom, H.

5   R. Bonnert, T. P., McCafferty, J., Griffiths, A. D. &

Winter, G. (1991) J. Mol. Biol. 222, 581-597)

2. <u>Synthetic library</u> (Nissim, A., Hoogenboom, H. R.,

Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D. and

10   Winter, G. (1994) EMBO J. 13, 692-698) derived from

cloned human germline VH genes and synthetic CDR3s

with a fixed light chain

3. <u>Tonsil library</u> derived from the tonsils of

15   unimmunised humans. Tonsil B cells were isolated from

freshly removed (processed within 2 hours) whole

tonsils provided by Addenbrookes Hospital, Hills Road,

Cambridge, U.K. Each tonsil was processed as follows.

Tonsils were placed in a petri dish containing 5ml of

20   PBS and macerated with a scalpel blade to release the

cells. The suspension was transferred to a fresh tube

and large debris allowed to sediment under gravity for

5 minutes. The cell suspension was then overlaid onto

10mls of Lymphoprep in a 50 ml polypropylene tube

25   (Falcon) and centrifuged at 1000xg 20 minutes at room

temperature (no brake) and cells at the interface

harvested with a glass pipette. These were diluted to

a final volume of 50 ml in RPMI medium at 37^0 C and

centrifuged at 500xg for 15 minutes at room

temperature. The supernatant was aspirated and the the

cells washed another two times with RPMI.

Polyadenylated RNA was prepared from pelleted

5   cells using the "QuickprepTM mRNA Kit" (Pharmacia

Biotech, Milton Keynes, U.K.). The entire output of

cells from one tonsil (ca. 1x10^6 cells) was processed

using one Oligo(dT)-Cellulose Spun column and

processed exactly as described in the accompanying

10   protocol. MRNA was ethanol precipitated as described

and resuspended in 40ml RNase free water.

The cDNA synthesis reaction was set up using the

"First-Strand cDNA Synthesis Kit (Pharmacia Biotech,

Milton Keynes, U.K.) as follows:

15   RNA   20μl (heated to 67°C 10

                                          minutes before use)

           1st strand buffer   11μl

           DTT solution   1μl

           pd(N)$_6$ primer   1μl

20   After gentle mixing, the reaction was incubated at

37°C for 1 hour.

Human VH genes were amplified from tonsil cDNA

using the nine family-based back primers (VH 1b/7a -6a

back Sfi , which introduce a Sfi I site at the 5'-end,

25   Table 1) together with an equimolar mixture of the

four JH forward primers (JH 1-2, 3, 4-5, 6, for; Marks

et al., 1991 supra). Thus, nine primary PCR

amplifications were performed. Each reaction mixture

(50 µl) comprised 2 µl cDNA template, 25 pmol back

primer, 25 pmol forward primers, 250 µM dNTPs, 1.5 mM

$MgCl_2$, 50 mM KCl, 10 mM Tris-HCL pH 8.3 and 2.5 u of

Taq polymerase (Boehringer). The reaction mixture was

5   overlaid with mineral (paraffin) oil and was cycled 30

times (94 ^{0}C for 1 min, 55 ^{0}C for 1 min, 72 ^{0}C for 1

min) using a Techne thermal cycler. The products were

purified on a 1% (w/v) agarose gel, isolated from the

gel using "Geneclean" (Bio 101 Inc.) and resuspended

10   in 15 µl of water. The amplified VH genes were

recombined with human VL genes derived from PBLs

(Marks et al., 1991 supra) together with the $(Gly_4,$

$Ser)_3$ linker (Huston, J.S., et al. 1988 Proc Natl Acad

Sci U S A. 85: 5879-83) by PCR assembly (Marks et al,

15   1991 supra). The VH-linker-VL antibody constructs were

cloned into the SfiI and NotI sites of the phagemid

vector, pCANTAB6 (McCafferty, J., et al. 1994 Appl.

Biochem. Biotech. 47: 157 - 173) to give a library of

6×10^7 clones.

20

4. <u>Large single chain Fv library</u> derived from lymphoid

tissues including tonsil, bone marrow and peripheral

blood lymphocytes.

Polyadenylated RNA was prepared from the B-cells

25   of various lymphoid tissues of 43 non-immunised donors

using the "Quickprep mRNA Kit" (Pharmacia).

First-strand cDNA was synthesized from mRNA using a

"First-strand cDNA synthesis" kit (Pharmacia) using

random hexamers to prime synthesis. V-genes were

amplified using family-specific primers for VH, Vκ and

Vλ genes as previously described (Marks et al., supra)

and subsequently recombined together with the (Gly$_4$,

5   Ser)$_3$ scFv linker by PCR assembly. The VH-linker-VL

antibody constructs were cloned into the *Sfi* I and *Not*

I sites of the phagemid vector, pCANTAB 6. Ligation,

electroporation and plating out of the cells was as

described previously (Marks et al, 1991 supra). The

10   library was made ca. 1000x larger than that described

previously by bulking up the amounts of vector and

insert used and by performing multiple

electroporations. This generated a scFv repertoire

that was calculated to have ca. 1.3 x 10^{10} individual

15   recombinants which by *Bst* NI fingerprinting were shown

to be extremely diverse.

a. Induction of phage antibody libraries

The four different phage antibody repertoires

20   above were selected for antibodies to TGFβ-1. The VH

synthetic (Nissim et al., 1994 supra), tonsil, 'large'

scFv and PBL (Marks et al., 1991 supra) repertoires

were each treated as follows in order to rescue

phagemid particles. 500 ml prewarmed (37 ^{0}C) 2YTAG

25   (2YT media supplemented with 100 μg/ml ampicillin and

2 % glucose) in a 2 l conical flask was inoculated

with approximately 3 x 10^{10} cells from a glycerol stock

(-70 ^{0}C) culture of the appropriate library. The

54

culture was grown at 37 °C with good aeration until the OD_{600nm} reached 0.7 (approximately 2 hours). M13K07 helper phage (Stratagene) was added to the culture to a multiplicity of infection (moi) of

5 approximately 10 (assuming that an OD_{600nm} of 1 is equivalent to 5×10^8 cells per ml of culture). The culture was incubated stationary at 37 °C for 15 minutes followed by 45 minutes with light aeration (200 rpm) at the same temperature. The culture was

10 centrifuged and the supernatant drained from the cell pellet. The cells were resuspended in 500 ml 2YTAK (2YT media supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin), and the culture incubated overnight at 30 °C with good aeration (300 rpm).

15 Phage particles were purified and concentrated by three polyethylene glycol (PEG) precipitations (Sambrook, J., Fritsch, E.F., & Maniatis, T. (1990). Molecular Cloning - A Laboratory Manual. Cold Spring Harbour, New York) and resuspended in PBS to 10^{12}

20 transducing units (tu)/ml (ampicillin resistant clones).

b. Panning of phage antibody library on TGFβ-1

Phage induced from the four repertoires were each

25 separately panned on TGFβ-1. A 75mm x 12mm immuno tube (Nunc; Maxisorp) was coated with 2 ml of recombinant human TGFβ-1 (0.5ug/ml, Genzyme) in PBS overnight at 4 °C. After washing 3 times with PBS, the tube was

filled with 3%MPBS (3 % 'Marvel' skimmed milk powder,

1x PBS) and incubated for 2 hours at 37 ^{0}C for

blocking. The wash was repeated, phagemid particles

(10^{13} tu) in 2 ml of 3% MPBS were added and the tube

5   incubated stationary at 37 ^{0}C for 1 hour. The tube

was washed 20 times with PBST(0.1%), then 20 times

with PBS. Bound phage particles were eluted from the

tube by adding 2 ml of 100mM-triethylamine, and

incubating the tube stationary at room temperature for

10   10 minutes. The eluted material was immediately

neutralised by pipetting into a tube containing 1 ml

1M-Tris.HCl (pH7.4). Phage were stored at 4 ^{0}C. 1.5

ml of the eluted phage were used to infect 20 ml of

logarithmically growing E. coli TG1 (Gibson, T.J.

15   (1984). PhD thesis. University of Cambridge, UK.).

Infected cells were grown for 1 hour at 37 ^{0}C with

light aeration in 2YT broth, and then plated on 2YTAG

medium in 243mm x 243mm dishes (Nunc). Plates were

incubated overnight at 30 ^{0}C. Colonies were scraped

20   off the plates into 10 ml of 2YT broth and 15 % (v/v)

glycerol added for storage at -70 ^{0}C.

Glycerol stock cultures from the first round of

panning of each of the four repertoires on TGFβ-1 were

each rescued using helper phage to derive phagemid

25   particles for the second round of panning. 250 μl of

glycerol stock was used to inoculate 50 ml 2YTAG

broth, and incubated in a 250 mL conical flask at 37

^{0}C with good aeration until the OD_{600mn} reached 0.7

(approximately 2 hours). M13K07 helper phage (moi=10)
was added to the culture which was then incubated
stationary at 37 °C for 15 minutes followed by 45
minutes with light aeration (200 rpm) at the same

5   temperature. The culture was centrifuged and the
supernatant drained from the cell pellet. The cells
were resuspended in 50 ml prewarmed 2YTAK, and the
culture incubated overnight at 30 °C with good
aeration. Phage particles were purified and

10 concentrated by PEG precipitation (Sambrook et al.,
1990 supra) and resuspended in PBS to 1013 tu/ml.

Phage induced from the first round of panning of
each of the three repertoires, was selected a second
time essentially as described above except that the

15 panning tube was coated with only 1 ml of TGFβ-1
(0.5ug/ml, Genzyme), and the volume of phage added to
the tube similarly reduced. After extensive washing,
bound phage were eluted from the tube using 1 ml of
100 mM-triethylamine, and neutralised by the addition

20 of 0.5 ml 1M-Tris.HCl (pH7.4) as earlier described.
The process of phage growth and panning was repeated
over a third and a fourth round of selection.

c. Growth of single selected clones for immunoassay

25   Individual colonies from the third and fourth
·round selections were used to inoculate 100 μl 2YTAG
into individual wells of 96 well tissue culture plates
(Corning). Plates were incubated at 30 °C overnight

with moderate shaking (200 rpm). Glycerol to 15 % was

added to each well and these master plates stored at

-70 ^{0}C until ready for analysis.

5   d. *ELISA to identify anti-TGFβ-1 scFv*

       Clones specific for TGFβ-1 were identified by

ELISA, using scFv displayed on phage or soluble scFv.

       i. Phage ELISA

10   Cells from the master plates were used to

inoculate fresh 96 well tissue culture plates

containing 100 μl 2YTAG per well. These plates were

incubated at 37 ^{0}C for 6-8 hours or until the cells in

the wells were growing logarithmically (OD600

15   0.2-1.0). M13K07 was added to each well to an moi of

10 and incubated stationary for 15 min then 45 min

with gentle shaking (100 rpm), both at 37 ^{0}C. The

plates were centrifuged at 2000 rpm for 10 min and the

supernatant eluted. Each cell pellet was resuspended

20   in 100 μl 2YTAK and incubated at 30 ^{0}C overnight.

       Each plate was centrifuged at 2000 rpm and the

100 μl supernatant from each well recovered and

blocked in 20 μl 18%M6PBS (18 % skimmed milk powder, 6

x PBS), stationary at room temperature for 1 hour.

25   Meanwhile, flexible microtitre plates which had been

blocked overnight stationary at 4 ^{0}C with either 50 μl

0.2 μg/ml TGFβ-1 in PBS or 50 μl PBS alone (giving an

uncoated control plate), were washed 3 times in PBS

and blocked for 2 h stationary at 37 ^{0}C in 3MPBS.
These plates were then washed three times with PBS and
50 μl preblocked phage added to each well of both the
TGFβ-1-coated or uncoated plate. The plates were

5   incubated stationary at 37 ^{0}C for 1 h after which the
phage were poured off. The plates were washed by
incubating for 2 min in PBST three·times followed by
incubating for 2min in PBS three times, all at room
temperature.

10   To each well of both the TGFβ-1-coated and the
uncoated plate, 50 μl of a 1 in 10,000 dilution of
sheep anti-fd antibody (Pharmacia) in 3MPBS was added
and the plates incubated at 37 ^{0}C stationary for 1 h.
·Each plate was washed as described above and 50 μl   of

15   a 1 in 5,000 dilution donkey anti-sheep alkaline
phosphatase conjugate (Sigma) in 3MPBS added and
incubated stationary at 37 ^{0}C for 1 h.· Plates were
washed as described as above followed by two rinses in
0.9% NaCl. Alkaline phosphatase activity was

20   visualised using either the chromagenic substrate pNPP
(Sigma) or the Ampak system (Dako). The absorbance
signal generated by each clone was assessed by
measuring the optical density at either 405 nm (pNPP)
or 492 nm (Ampak) using a microtitre plate reader.

25   Clones were chosen for further analysis if the ELISA
signal generated on the TGFβ-1-coated plate was at
least double that on the uncoated plate.

ii. Soluble ELISA

  Cells from the master plates were used to
inoculate fresh 96 well tissue culture plates
containing 100 μl 2YTAG per well. These plates were
5 incubated at 30 ^{0}C for 8 hours then centrifuged at
2000 rpm for 10 min and the supernatant eluted. Each
cell pellet was resuspended in 100 μl 2YTA (2YT media
supplemented with 100ug/ml ampicillin) containing 10
mM IPTG (isopropyl-B-D-thiogalactopyranoside) and
10 incubated at 30 ^{0}C overnight.

  Each plate was centrifuged at 2000 rpm and the
100 µl supernatant from each well recovered and
blocked in 20 µl 18%M6PBS stationary at room
temperature for 1 hour. Meanwhile, flexible
15 microtitre plates which had been blocked overnight
stationary at 4 ^{0}C with either 50 µl 0.2 µg/ml TGFβ-1
in PBS or 50 µl PBS alone, were washed 3 times in PBS
and blocked for 2 h stationary at 37 ^{0}C in 3%MPBS.
These plates were then washed three times with PBS and
20 50 µl preblocked soluble scFv added to each well of
both the TGFβ-1-coated or uncoated plate. The plates
were incubated stationary at 37 ^{0}C for 1 h after which
the scFv solutions were poured off. The plates were
washed by incubating for 2 min in PBST (PBS
25 containing 1% Tween) three times followed by
incubating for 2 min in PBS three times, all at room
temperature.

  To each well of both the TGFβ-1-coated and the

uncoated plate, 50 µl of a 1 in 200 dilution of the

anti-myc tag murine antibody 9E10 (Munro, S. & Pelham,

H.R.B. (1986)Cell 46, 291-300) in 3MPBS was added and

the plates incubated at 37 ^{0}C stationary for 1 h.

5   Each plate was washed as described above and 50 µl of

a 1 in 5,000 dilution goat anti-mouse alkaline

phosphatase conjugate (Pierce) in 3MPBS added and

incubated stationary at 37 ^{0}C for 1 h. Plates were

washed as described above followed by two rinses in

10   0.9% NaCl. Alkaline phosphatase activity was

visualised using either the chromagenic substrate pNPP

(Sigma) or the Ampak system (Dako). The absorbance

signal generated by each clone was assessed by

measuring the optical density at either 405 nm (pNPP)

15   or 492 nm (Ampak) using a microtitre plate reader.

Clones were chosen for further analysis if the ELISA

signal generated on the TGFβ-1-coated plate was at

least double that on the uncoated plate.

20   iii. Specificity ELISA

     Clones identified as binding TGFβ-1 rather an

uncoated well, as described above, were further

analysed for fine specificity. Specificity ELISA's

were carried out using scFv either displayed on phage

25   or in solution as described above, except that 5 ml of

media in 50 ml Falcon tubes were inoculated with each

clone and grown to generate the phage or soluble scFv

used in the ELISA. Microtitre plate wells were coated

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with 50 µl of either 0.2 µg/ml TGFβ-1, 0.2 µg/ml

TGFβ-2, 10 µg/ml bovine serum albumin (BSA) or PBS

(the uncoated well). After preblocking both the phage

(or soluble scFv) and the microtitre plates, 50 µl

5   blocked phage (or soluble scFv) from each clone was

added to a well coated with either TGFβ-1, TGFβ-2,

BSA or an uncoated well. As above, alkaline

phosphatse activity was visualised using either the

chromagenic substrate pNPP (Sigma) or the Ampak system

10   (Dako). Clones were considered to be specific for

TGFβ-1 if the ELISA signal generated in the TGFβ-1

coated well was at least five-fold greater than the

signal on either TGFβ-2, BSA or an uncoated well.

15   iv. Specificity determination by BIACore™

The antibodies were also shown to be specific for

TGFβ1 compared to TGFβ2 (obtained from R&D Systems

Abingdon) by relative binding to theBIACore™ sensor

chips coated with the appropriate antigen. TGFβ1 and

20   TGFβ2 were immobilised by amine coupling to Biosensor

CM5 sensorchips (Pharmacia) according to the

manufacturers instructions. Single chain Fv fragments

(35µl; purified by immobilized metal affinity

chromatography as described in example 4) were

25   injected over the immobilized antigen at a flow rate

of 5µl/min. The amount of TGFβ bound was assessed as

the total increase in resonance units (RUs) over this

period. For 31G9 scFv an increase of 1059RUs was found

62

with a TGFβ1 chip and 72 RUs was found with a TGFβ2

chip. Thus binding is much stronger to TGFβ1 than

TGFβ2.

5   *e. Sequencing of TGFb1-Specific ScFv Antibodies*

The nucleotide sequence of the TGFβ-1 specific

antibodies was determined by first using

vector-specific primers to amplify the inserted DNA

from each clone. Cells from an individual colony on

10   a 2YTAG agar plate were used as the template for a

polymerase chain reaction (PCR) amplification of the

inserted DNA using the primers pUC19reverse and

fdtetseq (Table 1). Amplification conditions

consisted of 30 cycles of 94 ^{0}C for 1 min, 55 ^{0}C for 1

15   min and 72 ^{0}C for 2 min, followed by 10 min at 72 ^{0}C.

The PCR products were purified using a PCR Clean-up

Kit (Promega) in to a final volume of 50 µl H20.

Between 2 and 5 µl of each insert preparation was used

as the template for sequencing using the Taq

20   Dye-terminator cycle sequencing system (Applied

Biosystems). The primers mycseq10 and PCR-L-Link were

used to sequence the light chain of each clone and

PCR-H-Link and pUC19reverse to sequence the heavy

chain (Table 1)

25

f. Sequence and Source of the Initial TGFβ-1-Specific

ScFv Antibodies

Four different TGFβ-1 specific antibodies were

isolated from the selections using the four libraries
described above. Each clone name, its origin and its
heavy and light chain germline is given below. The
complete sequence of the VH domain genes of clones 1-

5   B2 and 31-G9 are given in Figure 1(a) together with
the VL domain gene from scFv 31-G9.

CLONE	LIBRARY SOURCE	VH GERMLINE		VL ISOTYPE
1-B2	PBL	VH3	DP49	VKappa
1A-E5	Synthetic VH	VH3	DP53	VLambda
1A-H6	Tonsil	VH3	DP50	VLambda
31-G9	large scFv	VH3	DP49	VLambda

15   Thus these initial isolates were obtained from
libraries derived from different sources-both natural
V genes of unimmunised humans and synthetic libraries
from cloned germline V genes together with synthetic
CDRs.

20

2. Affinity Maturation of the Initial TGFβ-1-Specific
ScFv Antibodies

a. Light Chain Shuffling of the TGFβ-1-Specific ScFv

25   Antibody 1-B2

i. Construction of Repertoires
        The heavy chain of clone 1-B2 was recombined with

the complete repertoire of light chains derived from the PBL and large (tonsil-derived) scFv repertoires. The 1-B2 heavy chain was amplified by PCR using the primers HuJh4-5For (Table 1) and pUC19reverse.

5   Amplification conditions consisted of 30 cycles of 94 ^{0}C for 1 min, 55 ^{0}C for 1 min and 72 ^{0}C for 1min, followed by 10 min at 72 ^{0}C. The PCR product was separated through a 1% agarose-TAE gel, the band representing the amplified VH excised, and eluted from

10   the agarose gel using the Geneclean Kit (Bio 101).

The PBL and tonsil light chains were amplified by PCR using the primers fdtetseq and a mix of RL1, 2 & 3 (Table 1). Amplification conditions consisted of 30 cycles of 94 ^{0}C for 1 min, 55 ^{0}C for 1 min and 72 ^{0}C

15   for 1min, followed by 10 min at 72 ^{0}C. The PCR product was separated through a 1% agarose-TAE gel, the band representing the amplified VL excised, and eluted from the agarose gel using the Geneclean Kit (Bio 101).

20   Approximately 50 ng amplified 1-B2 heavy chain and 50 ng of either amplified PBL-derived or amplified tonsil-derived light chains were combined and precipitated with sodium acetate and ethanol using 25 μg glycogen as a carrier. The precipitated DNA was

25   pelleted by centrifugation at 13,000 rpm in a microfuge, air dried and resuspended in 26 μl H20. This was used in an assembly amplification after the addition of reaction buffer to 1X, dNTP's to 200 nM

and 5 units Taq polymerase. Amplification conditions
consisted of 20 cycles of 94 ^{0}C for 1 min, 60 ^{0}C for 1
min and 72 ^{0}C for 1min 30 s, followed by 10 min at 72
^{0}C. 10 μl of each assembly was used as the template

5   in a 'pull-through' amplification with the primers
fdtetseq and pUC19reverse. Amplification conditions
consisted of 25 cycles of 94 ^{0}C for 1 min, 60 ^{0}C for 1
min and 72 ^{0}C for 1min 30 s, followed by 10 min at 72
^{0}C.

10   The pull-through amplification product was
separated through 1% agarose-TAE and the band
representing the pull-through VH-VL excised and eluted
using the Geneclean Kit. This was digested with the
restriction endonucleases Sfi I and Not I (NEB) and

15   ligated (Amersham ligation system) into the phagemid
vector pCantab 6, previously digested with Sfi 1 and
Not I. The ligation product was used to transform
electrocompetent TG1 cells, plated out on 2YTAG plates
and incubated overnight at 30 ^{0}C. Approximately 1 x

20   10^5 individual clones were generated from the light
chain-shuffle of the 1-B2 heavy chain with the
PBL-derived light chains and approximately 1 x 10^6 for
the shuffle with the tonsil-derived light chains.

25   ii. Selection of Light Chain Shuffle Repertoires
          The two light chain-shuffle repertoires were
selected for TGFβ-1-specific antibodies. Phagemid
particles were recovered from each repertoire as

66

described earlier for the initial libraries.

Recovered phage were preblocked for 1 h in a final

volume of 100 μl 3MPBS. Approximately 10^{11} tu phage

were used in the first round selection and between 10^9

5   and 10^{10} for subsequent selections. For the first

round selections, biotinylated TGFβ1 to a final

concentration of 100 nM was added to the preblocked

phage and incubated stationary at 37^0C for 1h.

For each selection, 100 μl Dynabeads suspension

10   (Dynal) was separated on a magnet and the beads

recovered and preblocked for 2 h in 1 ml 3MPBS. The

beads were recovered on a magnet and resuspended in

the phagemid/biotinylated TGFβ-1 mixture and incubated

at room temperature for 15 min while being turned

15   end-over-end. The beads were captured on a magnet and

washed four times with PBST followed by three washes

in PBS. After each wash, the beads were captured on a

magnet and resuspended in the next wash. Finally,

half of the beads were resuspended in 10 μl 50 mM DTT

20   (the other half of the beads stored at 4 ^{0}C as a

back-up) and incubated at room temperature for 5 min.

The whole bead suspension was then used to infect 5 ml

logarithmically-growing TG1 cells. This was incubated

at 37 ^{0}C, stationary for 15 min then with moderate

25   shaking for 45 min, plated on 2YTAG plates and

incubated overnight at 30 ^{0}C.

Colonies were scraped off the plates into 10 ml

of 2YT broth and 15 % (v/v) glycerol added for storage

at -70 ^{0}C. A 250 µl aliqout of each plate scrape was used to inoculate 2YTAG and phagemid particles rescued as described earlier. For each repertoire, three rounds of selection using biotinylated TGFβ-1 was

5 performed, essentially identical to the first round selection described above. All selections were at 100 nM TGFβ-1 except for the third round selection of the tonsil-derived light chain repertoire where the concentration of biotinylated TGFβ-1 in the selection

10 was reduced to 50 nM.

iii. Identification of TGFβ-1-Specific ScFv Antibodies from Light Chain Shuffle Repertoires

ScFv antibodies specific to TGFβ-1 were

15 identified by both phage and soluble ELISA, and sequenced, as described earlier. Three new TGFβ-1-specific scFv antibodies were identified, two with PBL-derived light chains and one with a tonsil-derived light chain. All three had the 1B2

20 heavy chain sequence (DP49), described earlier. The sequences are summarised below and the complete sequence of each VL domain gene is given in figure 1(b).

25	CLONE	VL SOURCE	VH GERMLINE	VL ISOTYPE
	7-A3	PBL	DP49 (1B2)	VKappa
	10-A6	PBL	DP49 (1B2)	VLambda

14-A1   Tonsil   DP49 (1B2)   VLambda

Thus the VH domain 1B2 derived from the PBL

library can be combined with VL domains derived from

5   both PBL and tonsil libraries.

b. *CDR3 'Spiking' of the TGFβ-1-Specific ScFv Antibody*
1B2

10 i. Construction of 'spiked' repertoire

An 84 mer mutagenic oligonucleotide primer, 1B2

mutVHCDR3, was first synthesized (see Table 1). This

primer was 'spiked' at 10%; i.e. at each nucleotide

position there is a 10% probability that a

15 non-parental nucleotide will be incorporated. The

1-B2 heavy chain was amplified by PCR using the

primers pUC19reverse and 1B2 mutVHCDR3. Amplification

conditions consisted of 30 cycles of 94 ^{0}C for 1 min,

55 ^{0}C for 1 min and 72 ^{0}C for 1min, followed by 10 min

20 at 72 ^{0}C. The PCR product was separated through a 1%

agarose-TAE gel, the band representing the amplified

VH excised, and eluted from the agarose gel using the

Geneclean Kit (Bio 101).

The parental 1B2 light chain was amplified by PCR

25 using the primers fdtetseq and RL3 (Table 1).

Amplification conditions consisted of 30 cycles of 94

^{0}C for 1 min, 55 ^{0}C for 1 min and 72 ^{0}C for 1min,

followed by 10 min at 72 ^{0}C. The PCR product was

separated through a 1% agarose-TAE gel, the band
representing the amplified VL excised, and eluted from
the agarose gel using the Geneclean Kit (Bio 101).

5   Approximately 50 ng amplified 'spiked' 1-B2 heavy
chain and 50 ng of amplified parental 1B2 light chain
were combined and precipitated with sodium acetate and
ethanol using 25 μg glycogen as a carrier. The
precipitated DNA was pelleted by centrifugation at
13,000 rpm in a microfuge, air dried and resuspended
10   in 26 μl H20. This was used in an assembly
amplification after the addition of reaction buffer to
1X, dNTP's to 200 nM and 5 units Taq polymerase.
Amplification conditions consisted of 25 cycles of 94
^{0}C for 1 min, 65 ^{0}C for 4 min. Five μl of each
15   assembly was used as the template in a 'pull-through'
amplification with the primers fdtetseq and
pUC19reverse. Amplification conditions consisted of
30 cycles of 94 ^{0}C for 1 min, 55 ^{0}C for 2 min and 72 ^{0}C
for 1min, followed by 10 min at 72 ^{0}C.

20 .   The pull-through amplification product was
separated through 1% agarose-TAE and the band
representing the pull-through 'spiked' VH -VL excised
and eluted using the Geneclean Kit. This was digested
with the restriction endonucleases Sfi I and Not I
25   (NEB) and ligated (Amersham ligation system) into the
phagemid vector pCantab 6, previously digested with
Sfi I and Not I. The ligation product was used to
transform electrocompetent TG1 cells, plated out on

70

2YTAG plates and incubated overnight at 30 ^{0}C.

Approximately 4 x 10^6 individual clones were generated

from this VH CDR3 'spiking' of the 1-B2 VH CDR3.

5   ii. Selection of 1B2 CDR3 Spike Repertoire

The repertoire was selected for new

TGFβ-1-specific scFv antibody by one round of panning

on 1 µg/ml TGFβ-1 followed by two rounds of selection

with biotinylated TGFβ-1 at 50 nM using methods as

10   described earlier.

iii. Identification of TGFβ-1-Specific ScFv Antibodies

from the 1B2 CDR3 Spike Repertoire

ScFv antibodies specific to TGFβ-1 were

15   identified by both phage and soluble and phage ELISA,

and sequenced, as described earlier. Clone 27C1 was

isolated from the spiked repertoire. It is virtually

identical to clone 1B2 but with three differences in

the heavy chain CDR3. The complete sequence of clone

20   27C1 is given in figure 1 (c). The 27C1 VH domain was

combined with the 10A6 VL domain in the construction

of the whole antibody 27C1/10A6 IgG4 (example 2). The

properties of this antibody are described in more

detail in examples 2 to 6. In addition to 27C1, a

25   large number of other antibodies were isolated with up

to 7 of the 14 amino acids differing in CDR3 of the VH

domain (Figure 3). These had a similar preference for

binding TGFβ1 compared to TGFβ2.

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3.   Identification and Characterisation of Antibodies

to Human TGFβ-2 by Selection of Naive and Synthetic

Phage Antibody Repertoires

5   a. Induction of phage antibody libraries

Two different phage antibody repertoires were

selected for antibodies to TGFβ-2. The VH synthetic

(Nissim et al., 1994) and tonsil (constructed as

described earlier) repertoires were each treated as

10   described for TGFβ-1 to rescue phagemid particles.

b. Panning of phage antibody library on TGFβ-2

Phage induced from the two repertoires were each

separately panned on TGFβ-2 as described earlier for

15   TGFβ-1 but using 0.5 µg/ml TGFβ-2 as the coating.

antigen.

c. Identification and Sequencing of TGFβ-2-Specific

ScFv Antibodies

20   Individual colonies from the third and fourth

round selections were screened by both phage and

soluble ELISA as described earlier for TGFβ-1 but

using flexible microtitre plates coated with TGFβ-2 at

0.2 µg/ml rather than TGFβ-1. Clones were chosen for

25   further analysis if the ELISA signal generated on the

TGFβ-2-coated plate was at least double that on the

uncoated plate. For the specificity ELISA, as

described earlier for TGFβ-1, clones were considered

to be specific for TGFβ-2 if the ELISA signal

generated in the TGFβ-2 coated well was at least

five-fold greater than the signal on either TGFβ-1,

BSA or an uncoated well.

5

d. Sequence and Source of the Initial TGFβ-2-Specific

ScFv Antibodies

        Four different TGFβ-2 specific antibodies were

isolated from the selections using the two libraries

10   described above. Each clone name, its origin and its

heavy and light chain germline is given below. The

complete sequence of the VH domain genes of 2A-H11 and

2A-A9 are given in Figure 2 (a).

15	CLONE	LIBRARY SOURCE	VH GERMLINE	VL ISOTYPE
	1-G2	Tonsil		
	1-H6	Tonsil	DP49	
	2A-H11	Synthetic VH	DP50	VLambda
20	2A-A9	Synthetic	DP46	VLambda
	Gold-11	Large scFv		VLambda

        Thus human antibodies binding to human TGFβ2 have

been isolated from different sources-, both natural

25   Vgenes of unimmunised humans and synthetic libraries

from cloned germline V genes together with synthetic

CDRs.

4. Light Chain Shuffling of the TGFβ-2-Specific ScFv
Antibodies 2A-H11 and 2A-A9

a. Construction of Repertoires

5   The heavy chain of clones 2A-H11 and 2A-A9 were

recombined with the complete repertoire of light

chains derived from the PBL and large (tonsil-derived)

scFv repertoires as described earlier for the

TGFβ-1-specific scFv antibody 1-B2. Both repertoires

10   generated from the recombination with the PBL light

chain repertoire were approximately 1×10^5, those

generated from the recombination with the tonsil light

chain repertoire were approximately 1×10^6.

15   b. Selection of Light Chain Shuffle Repertoires

       The light chain-shuffle repertoires were selected

for TGFβ-2-specific antibodies using biotinylated

TGFβ-2, as described earlier for the selection of the

TGFβ-1 light chain shuffle repertoires. For all of

20   the first and second round selections, a

concentrartion of 100 nM biotinylated TGFβ-2 was used.

For the third round selection of the PBL-derived light

chain shuffle repertoire, biotinylated TGFβ-2 was used

at concentrations of 100 nM and 1 nM. For the third

25   round selection of the tonsil-derived light chain

shuffle repertoire, biotinylated TGFβ-2 was used at a

concentration of 50 nM.

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c. Identification of TGFβ-2-Specific ScFv Antibodies
from Light Chain Shuffle Repertoires

ScFv antibodies specific to TGFβ-2 were
identified by both phage and soluble ELISA, and
5   sequenced, as described earlier. Five new
TGFβ-2-specific scFv antibodies were identified. The
sequences are summarised below and the complete
sequence of each clone given in Figure 2 (b).

10	CLONE	VL SOURCE	VH GERMLINE	VL ISOTYPE
	6-H1	PBL	DP50 (2A-H11)	VKappa
	6-A5	PBL	DP50 (2A-H11)	VLambda
	6-B1	PBL	DP50 (2A-H11)	VLambda
15	11-E6	PBL	DP46 (2A-A9)	VKappa
	14-F12	Tonsil	DP46 (2A-A9)	VLambda

d. Specificity determination by ELISA

Clones identified as binding TGFβ-2 rather an
20   uncoated well, as described above, were further
analysed for fine specificity. Specificity ELISA's
were carried out using scFv either displayed on phage
or in solution as described above, except that 5 ml of
media in 50 ml Falcon tubes were inoculated with each
25   clone and grown to generate the phage or soluble scFv
used in the ELISA. Microtitre plate wells were coated
with 50 μl of either 0.2 μg/ml TGFβ-1, 0.2 μg/ml
TGFβ-2, 10 μg/ml bovine serum albumin (BSA) or PBS

(the uncoated well). After preblocking both the phage
(or soluble scFv) and the microtitre plates, 50 μl
blocked phage (or soluble scFv) from each clone was
added to a well coated with either TGFβ-1, TGFβ-2, BSA

5   or an uncoated well. As above, alkaline phosphatse
activity was visualised using either the chromagenic
substrate pNPP (Sigma) or the Ampak system (Dako).
Clones were considered to be specific for TGFβ-2 if
the ELISA signal generated in the TGFβ-2 coated well

10   was at least five-fold greater than the signal on
either TGFβ-1, BSA or an uncoated well.
Cross-reactivity with unrelated antigens was
determined more extensively for anti-TGFβ2 antibody in
whole antibody format, see example 2. The

15   cross-reactivity of 6B1 IgG4 and 6A5 IgG4 with TGFβ1
and TGFβ3 (obtained from R&D Systems, Abingdon) is
also shown to be very low.

e. Specificity determination by BIACore™

20   The antibodies were also shown to be specific for
TGFβ2 compared to TGFβ1 by relative binding to
theBIACore sensor chips coated with the appropriate
antigen. TGFβ1 and TGFβ2 were immobilised by amine
coupling to Biosensor CM5 sensorchips (Pharmacia)

25   according to the manufacturers instructions. Single
chain Fv fragments (35ul; purified by immobilized
metal affinity chromatography) were injected over the
immobilized antigen at a flow rate of 5μl/min. The

amount of TGFβ bound was assessed as the total

increase in resonance units (RUs) over this period.

For the single chain Fv fragments 6H1, 6A5 and 14F12,

these fragments gave a total of 686, 480 and 616 RUs

5   respectively for the TGFβ1 coated sensor chip and 77,

71 and 115 RUs respectively for the TGFβ2 coated chip.

*5.   Building higher affinity anti TGFβ-1 biological
neutralisers*

10

a. Recombining heavy chains derived from high affinity
anti- TGFβ1 scFv with light chains derived from anti
-TGFβ1 and anti-TGFβ2 scFv showing good properties

      Antibodies derived by spiking CDR3 of the scFv

15   antibody 1-B2 (section 2b) bind TGFβ-1 with high

affinity. To improve the chance of obtaining high

affinity neutralising antibodies it was decided to

chain shuffle VHs derived from high affinity

anti-TGFβ-1 scFv with VLs derived from scFv clones

20   with promising properties and particularly with those

capable of neutralising the activity of TGFβ-2 in

vitro.

      Heavy chains were amplified by PCR from the

repertoire of CDR3 spiked 1-B2 clones after selection

25   on TGFβ-1(section 2a.ii) using the primers

pUC19reverse and PCR-H-Link (Table 1). Amplification

conditions consisted of 30 cycles of 94 ^{6}C for 1 min,

55 ^{0}C for 1 min and 72 ^{0}C for 1min, followed by 10 min

77

at 72 ^{0}C. The PCR product was separated through a 1%

agarose-TAE gel, the band representing the amplified

VH excised, and eluted from the agarose gel using the

Geneclean Kit (Bio 101).

5   Light chains were separately amplified by PCR

from each of the anti TGFβ-1 specific neutralisers (

7-A3, 10-A6 and 14-A1; section 2a.iii) and each of the

anti TGFβ-2 specific neutralisers (6H1, 6A5, 6B1, 11E6

and 14F12; section 4c) using the primers fdtetseq1

10   and PCR-L-Link (Table 1). The same PCR conditions

were used as described for theVH amplification. Each

VL PCR product was then separately purified through a

1% agarose-TAE gel as described above. Purified

products were finally mixed in approximately equimolar

15   amounts (as estimated from an analytical agarose gel)

to provide a VL 'pool'.

Approximately 50 ng amplified heavy chains and 50

ng of amplified pooled light chains were combined and

precipitated with sodium acetate and ethanol using 25

20   μg glycogen as a carrier. The precipitated DNA was

pelleted by centrifugation at 13,000 rpm in a

microfuge, air dried and resuspended in 23 μl H20.

This was used in an assembly amplification after the

addition of reaction buffer, dNTP's to 200 nM and 5

25   units Taq polymerase. Amplification conditions

consisted of 20 cycles of 94 ^{0}C for 1 min, 55 ^{0}C for 1

min and 72 ^{0}C for 2 mins, followed by 10 min at 72 ^{0}C.

5 μl of assembly was used as the template in a 50ul

78

'pull-through' amplification with the primers fdtetseq

and pUC19reverse. Amplification conditions consisted

of 30 cycles of 94 ^{0}C for 1 min, 55 ^{0}C for 1 min and 72

^{0}C for 2mins, followed by 10 min at 72 ^{0}C.

5   The pull-through amplification product was

separated through 1% agarose-TAE and the band

representing the pull-through VH-VL excised and eluted

using the Geneclean Kit. This was digested with the

restriction endonucleases Sfi I and Not I (NEB) and

10   ligated into the phagemid vector pCantab 6 (McCafferty

et al. 1994 supra), previously digested with Sfi 1 and

Not I, using the Amersham ligation system. The

ligation product was used to transform

electrocompetent TG1 cells, plated out on 2YTAG plates

15   and incubated overnight at 30 ^{0}C. A repertoire of

approximately 3 x 10^6 individual clones was generated.

b. Selection of chain shuffled repertoire

       The chain shuffled repertoire was selected by a

20   single round of panning on TGFβ-1 (1ug/ml), as

previously described (section 1b).

c. Identification of TGFβ-1 specific scFv antibodies

       ScFv antibodies specific to TGFβ-1 were

25   identified by phage ELISA and sequenced as described

earlier (sections 1d.i and 1e). New TGFβ-1 specific

scFv antibodies were identified. Five new high

affinity clones were isolated - CS32 which comprises

31G9 VH and 7A3 VL; CS39 which comprises 31G9 VH and
6H1 VL; CS37 which comprises 31G9 VH Figure 1(a) (iii)
and 11E6 VL with an Ile for Val substitution at
residue 2 (VL sequence given in Figure 14); CS35 which

5   comprises 31G9 heavy chain with substitutions of Glu
for Gln at residue 1, Gln for Glu at residue 5 and
14F12 VL; and CS38 which comprises 31G9 VH with
substitutions of Thr for Gln at residue 3, Glu for Gln
at residue 5, Leu for Phe at residue 27, Ile for Asn

10   at residue 56 and Arg for Gln at residue 105 and 6A5
VL.

d. Off-rate determination for single chain Fv
fragments binding to TGFβ1 and TGFβ2

15   The off-rates for binding to TGFβ1 or TGFβ2 of
the single chain Fv fragments described in this
example were determined as described by Karlsson et al
(R. Karlsson et al, J. Immunol. Methods 145, 229-240,
1991). The results obtained are shown in Table 2,

20   together with dissociation constants for those which
have been determined. These results indicate that high
affinity antibodies have been isolated.

6. Identification and Characterisation of an Antibody

25   which Cross-reacts with both Human TGFβ-1 and TGFβ-2
but not TGFβ-3 by Selection of a Large ScFv Repertoire

a. Panning of the Library and Identification of

<u>Binders</u>

     The large scFv library (described earlier) was

induced, phagemid particles rescued and panned as

described earlier with the following modifications.

5   For the first round of panning, 10^{12} tu library phage

in 0.5 ml PBS were used (rather than the standard 2

ml), for the second round, 3.5×10^9 phage in 0.5 ml

PBS were used. The immuno tube was coated with 10 µg

TGFβ-2 in 0.5 ml PBS for both the first and second

10   round of selection. Individual colonies from the

second selection were screened by ELISA using 0.2

µg/ml TGFβ-1. Clones binding TGFβ-1 were further

screened on TGFβ-2, TGFβ-3, BSA and PBS. Clones were

considered to be specific for both TGFβ-1 and TGFβ-2

15   if the ELISA signal generated in the TGFβ-1 and the

TGFβ-2 coated wells were both at least five-fold

greater than the signal on TGFβ-3, BSA and an uncoated

well.

20   <u>c. Identification of a TGFβ-1/TGFβ-2 Cross-reactive</u>

<u>ScFv Antibody</u>

     A single scFv antibody specific for both TGFβ-1

and TGFβ-2 was identified by both phage and soluble

ELISA, and sequenced, as described earlier. The

25   complete sequence of the VL domain of the antibody

gene VT37 is given in figure 4. The dissociation

constant of this single chain Fv antibody was

estimated by analysis using BIACore™ to be 4nM for

TGFβ1 and 7nM for TGFβ2. Cross-reactivity for TGFβ3

was also determined. Purified VT37scFv at 8.3μg/ml was

passed over BIACore™ sensor chips coated with TGFβ1

(500RUs coated); TGFβ2 (450RUs coated) or TGFβ3

5   (5500RUs coated). The relative response for VT37 scFv

binding was: TGFβ1 - 391RU bound; TGFβ2 - 261RU bound

or TGFβ3 - 24RU bound. Thus this antibody binds

strongly to TGFβ1 and TGFβ2 but binding to TGF β 3 is

not detectable above background.

10

EXAMPLE 2

Construction of Cell Lines Expressing Whole Antibodies

        For the construction of cell lines expressing

IgG4 antibodies, variable domains were cloned into

15   vectors expressing the human gamma 4 constant region

for the VH domains or the human kappa or lambda

constant regions for the VL domains.

        To construct the whole antibody, 27C1/10A6 IgG4

(specific for TGFβ$_1$), 27C1 VH DNA was prepared from

20   the clone isolated above, in example 1. The VH gene

was amplified by PCR using the oligonucleotides

VH3BackSfiEu and VHJH6ForBam (Table 1) with cycles of

1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C.

Following digestion with SfiI and BamHI, the VH gene

25   was cloned into the vector vhcassette2 (Figure 5)

digested with SfiI and BamHI. Ligated DNA was

transformed into E. coli TG1. Ampicillin resistant

colonies were obtained and those containing the

correct insert identified by DNA sequencing.

Plasmid DNA from these colonies was prepared and
the DNA digested with HindIII and BamHI. The
HindIII-BamHI restriction fragment was ligated into
5   the human IgG4 heavy chain expression vector pG4D100
(Figure 6), which had been digested with HindIII and
BamHI and the DNA transfected into E.coli TG1 by
electroporation. The sequence of the VH gene insert
was again verified by DNA sequencing.

10   For the light chain, the VL gene of 10A6,
isolated in example 1, was first mutagenized to remove
its internal BamHI site using site directed
mutagenesis (Amersham RPN1523) with the
oligonucleotide DeltaBamHI (Table 1). The resulting
15   VLDBamH1 gene was amplified by PCR using the
oligonucleotides Vλ3/4BackEuApa and HuJλ2-3ForEuBam
(Table 1). Following digestion of the amplified insert
with ApaLI and BamHI, the VL gene was cloned into the
vector vlcassetteCAT1 (Figure 7) digested with ApaLI
20   and BamHI. Ligated DNA was transformed into E.coli
TG1. Ampicillin resistant colonies were obtained and
those containing the correct insert were identified by
DNA sequencing.

Plasmid DNA from these colonies was prepared and
25   the DNA digested with Hind III and BamHI. The
HindIII-BamHI restriction fragment containing the
leader sequence and the VL domain was ligated into the
human lambda light chain expression vector, pLN10

(Figure 8), which had been digested with HindIII and
BamHI. Following electroporation, transformants in
E.coli were checked by DNA sequencing.

Plasmid DNA was prepared from the pG4D100-27C1

5   clone and the pLN10-10A6 clone. This DNA was then
co-transfected into DUKXB11 Chinese Hamster Ovary
(CHO) cells by electroporation (290V; 960µF). The
cells were then grown for 2 days in non-selective
medium (alpha-MEM plus nucleosides). Cells were then

10   transferred to a selective medium (alpha-MEM plus
1mg/ml G418 without nucleosides) and grown in 96 well
plates. Colonies were then transferred to 24 well
plates and samples assayed by sandwich ELISA for
assembled human IgG4 antibody and by binding to TGFβ1

15   in ELISA (as in example 1). For the sandwich ELISA,
goat anti-human IgG coated on to the ELISA plate and
captured human IgG4 detected using goat antihuman
lambda light chain alkaline phosphatase conjugate.
High expressing cell lines were then derived by

20   amplification of the inserted genes using selection in
the presence of methotrexate (R.J. Kaufman Methods
Enzymol. 185 537-566, 1990).

The whole antibody 6H1 IgG4 (specific for TGFβ2)
was constructed in a similar way to the above

25   construction of 27C1/10A6 IgG4. The 6H1 VH gene
(example 2) was cloned into pG4D100 as for 27C1 above
except that PCR amplification was performed with the
oligonucleotides VH3BackSfiEu and VHJH1-2FORBam. The

6H1 VL gene (example 2) was subcloned into
vlcassetteCAT1 as above except that PCR amplification
was performed with the oligonucleotides Vk2BackEuApa
and HuJk3FOREuBam. However, since the 6H1 VL is a

5   kappa light chain the HindIII-BamHI fragment was
subcloned into the human kappa light chain expression
vector pKN100 (Figure 9) which had been digested with
HindIII and BamHI. High expressing cell lines were
then isolated as described above. Clones expressing

10   antibody were identified from culture plates by
sandwich ELISA for assembled human IgG4 antibody
(detected using goat anti-human kappa light chain
conjugate and by binding to TGFβ2 in ELISA (as in
example 2).

15   To construct the whole antibodies 6A5 IgG4 and
6B1 IgG4, the same 6H1 VH construct in pG4D100 was
used as for 6H1IgG4 since these antibodies all have
the same VH gene. The 6B1 and 6A5 genes were each
subcloned into vlcassetteCAT1 as above for the 10A6

20   light chain except that PCR amplification was
performed with the nucleotides Vλ3backEuApa and
HuJλ2-3ForEuBam. The HindIII-BamHI restriction
fragment was then subcloned into pLN10 as above.
Clones expressing antibody were identified from

25   culture plates by sandwich ELISA for assembled human
IgG4 antibody (detected using goat anti-human kappa
light chain conjugate and by binding to TGFβ2 in ELISA
(as in example 2).

Properties of whole antibody constructs

<u>Purification of whole antibodies</u>

     Serum-free supernatant from CHO cells producing

5   the relevant IgG was clarified by centrifugation at

8000 rpm (Beckman JS2-21) prior to purification. The

supernatant was applied to a HiTrap Protein A

Sepharose prepacked affinity column from Pharmacia,

either 1 or 5ml size, with binding capacities of 25 or

10   120 mg respectively. Each IgG had a dedicated column

to avoid any potential carry over of material from one

purification to another. The column was equilibrated

to phosphate buffered saline (PBS) with ten column

volumes of 1xPBS prior to applying the supernatant.

15   When all the supernatant had been applied to the

column at a flow rate of 2-4 ml/minute, again,

depending on the column size, the column was washed

with ten column volumes of 1xPBS to remove any

non-specifically bound material. Elution of the bound

20   protein was achieved using 0.1M sodium acetate,

adjusted to pH 3.3 with glacial acetic acid. The

eluted material was collected in 8 fractions of 1.5 ml

volume, and the amount of protein determined by

measuring the absorbance at 280nm, and multiplying

25   this value by 0.7 to get a value in mg/ml. This was

then neutralised with 0.5ml of 1M Tris.HCl pH 9.0 per

1.5ml fraction, and the protein-containing fractions

pooled and dialysed against 1x PBS to buffer exchange

the IgG. The column was returned to neutral pH by

running ten column volumes of 1xPBS through, and was

stored in 20% ethanol as a preservative until required

again.

5   A sample was then run on 10-15% SDS-PAGE (Phast

system, Pharmacia) and silver stained. this allowed

an assessment of the purity of the IgG preparation.

This was usually found to be about 80-90%, with only a

couple of other bands prominent on the stained gel.

10

Binding specificity by ELISA

        The IgG4 antibodies 6B1 and 6A5 were shown to

bind TGFβ2 with very low cross-reactivity to TGFβ1 and

TGFβ3 and no detectable cross-reactivity with a range

15 of non-specific antigens: interleukin-1; human

lymphotoxin (TNFb); human insulin; human serum

albumin; single stranded DNA; oxazolone-bovine serum

albumin; keyhole limpet haemocyanin; chicken egg white

trypsin inhibitor; chymotrypsinogen; cytochrome c;

20 glyceraldehyde phosphate dehydrogenase; ovalbumin; hen

egg lysozyme; bovine serum albumin and tumour necrosis

factor a - (TNFa) (Figure 13(a) and (b)). Likewise the

antibodies 6B1, 6A5 and 6H1 IgG4 bound strongly to

TGFβ2 coated on a BIACore™ sensor chip but not

25 significantly to TGFβ1 or TGFβ3 coated chips.

Binding properties of whole antibodies by BIACore™

        The affinity constants of the above antibodies

were determined by BIACore™, using the method of

Karlsson et al. J. Immunol. Methods 145, 299-240, 1991

(supra) and found to be approximately 5nM for

27C1/10A6 IgG4 for TGFβ1 and 2nM for 6H1 IgG4 for

5   TGFβ2. The antibody 27C1/10A6 IgG4 also shows some

cross-reactivity with TGFβ2 coated onto Biosensor

chips but the dissociation constant is approximately

10 fold or more higher for TGFβ2 compared to TGFβ1.

There was no significant cross-reactivity with

10   lysozyme coated onto a BIACore™ sensor chip.

Neutralisation and inhibition of radioreceptor

binding by IgG4 antibodies to TGFβ1 and TGFβ 2 is

described in examples 3 and 4.

15   *EXAMPLE 3*

Neutralisation by Antibodies of the Inhibitory Effect
of TGF β1 and TGF β2 on Cell Proliferation

The neutralising activity of the antibodies

described in examples 1 and 2 were tested in a

20   modification of a bioassay for TGF β as described by

Randall et al (1993) J. Immunol Methods 164, 61-67.

This assay is based on the ability of TGF β_1 and TGF β_2

to inhibit the interleukin-5 induced proliferation of

the erythroleukaemia cell line, TF1 and being able to

25   reverse this inhibition with specific TGF β

antibodies.

Method

Cells and maintenance

The human erythroleukaemia cell line TF1 was

grown in RPMI 1640 medium supplemented with 5% foetal

calf serum, penicillin/streptomycin and 2ng/ml

5   rhGM-CSF in a humidified incubator containing 5% CO_2

at 37°C. Cultures were passaged when they reached a

density of 2 X 10^5/ml and diluted to a density of 5 x

10^5/ml.

10   Cytokines and Antibodies

rhGM-CSF and rhIL-5 were obtained from R&D

systems, rhTGF β_2 was obtained AMS Biotechnology.

Rabbit anti TGF β_2 antibody was from R&D Systems and

Mouse anti-TGF $\beta_{1,2,3}$ was from Genzyme. Other

15   antibodies against TGF β_2 were as described in

examples 1&2.

Titration of Inhibition of Proliferation by TGF β_2.

Doubling dilutions of TGF β_2 (800pM - 25pM) for

20   the construction of a dose response curve were

prepared on a sterile microtitre plate in 100μl of

RPMI 1640 medium containing 5% foetal calf serum and

antibiotics. All dilutions were performed at least in

quadruplicate. Additional wells containing 100μl of

25   the above medium for reagent and cells only controls

were also included.

TF1 cells were washed twice in serum free RPMI

1640 medium and resuspended in RPMI 1640 medium

supplemented with 5% foetal calf serum, 100U/ml

penicillin and 100µg/ml streptomycin and 4ng/ml rhIL-5

at a density of 2.5 x 10^5/ml. Aliquots of 100µl were

added to the previously prepared dilution series and

5   the plate incubated for 48hr. in a humidified

incubator containing 5% CO_2 at 37°C.

Cell proliferation was measured colourimetrically

by addition of 40µl CellTiter96 substrate (Promega),

returning the plate to the incubator for a further 4hr

10   and finally determining the absorbance at 490nm. The

percentage inhibition for each concentration of TGF β_2

as compared to cell only wells was then calculated.

Assay for Neutralisation of TGF β_2 Inhibitory Activity

15   by Anti-TGF β_2 Antibodies

Neutralisation of TGF β_2 was determined by making

doubling dilutions in of each purified antibody in

100µl of medium as above. TGF β_2 was added to each

antibody dilution to give a final concentration

20   equivalent to that which gave 50% inhibition in the

titration described above. Each dilution was prepared

in quadruplicate. Additional wells were prepared for

antibody only, cells only and reagent controls. Cell

preparation and determination of cell proliferation

25   was performed as described above.

Results

TGF β_2 was shown to inhibit the proliferation of

TF1 cells by 50% at a concentration of 50pM. This

concentration was used for all neutralisation

experiments.

These assays showed that TGF β_2 activity was

5   neutralised in a dose dependant manner for both scFv

fragments (figure 10) and for whole IgG4 antibodies

(figure 11). The concentration of antibody which gave

50% inhibition was determined from the graphs and is

shown in table 4.

10

EXAMPLE 4

Inhibition by Antibodies of TGFβ Binding to Receptors

Measured in A Radioreceptor Assay

Single chain Fv fragments and whole IgG4

15   antibodies from different clones were expressed and

purified and their ability to inhibit binding of TGFβ

to receptors measured in a radioreceptor assay.

Purification of scFv

20   ScFvs containing a poly histidine tail are

purified by immobilised metal affinity chromatography.

The bacterial clone containing the appropriate plasmid

is inoculated into 50 ml 2TY medium containing 2%

glucose and 100 µg/ml ampicillin (2TYAG) and grown

25   overnight at 30°C. The next day the culture is added

to 500 ml prewarmed 2TYAG and grown at 30°C for 1 h.

The cells are collected by centrifugation and added to

500 ml 2TY containing ampicillin and 1 mM IPTG and

grown at 30°C for 4 h. The cells are then collected

by centrifugation and are resuspended in 30 ml

ice-cold 50 mM Tris HCl pH 8.0, 20% (w/v) sucrose, 1

mM EDTA. After 15 min end-to-end mixing at 4°C the

5   mixture is centrifuged at 12 k rpm for 15 min at 4°C.

The supernatant is removed and to it added ~ 1ml

NTA-agarose (Qiagen 30210) and mixed at 4°C for 30

min.   The agarose beads are washed extensively with

50 mM sodium phosphate, 300 mM NaCl and loaded into a

10   small column. After further washing with 50 mM sodium

phosphate, 300 mM NaCl, 10 mM imidazole pH 7.4 scFv is

eluted with 50 mM sodium phosphate, 300 mM NaCl, 250

mM imidazole pH 7.4. 0.5 ml fractions are collected

and the protein containing fractions identified by

15   measuring the A_{280nm}. Pooled fractions are

concentrated and scFv further purified by gel

filtration in PBS on a Superdex 75 column (Pharmacia).

Purification of Whole Antibodies

20   Whole IgG4 antibodies were purified as described

in Example 2.

Radioreceptor Assay for TGF-β

Neutralisation of TGF-β activity is measured by

25   the ability of the scFvs and IgGs to inhibit the

binding of [125]-I labelled TGF-β to its receptors on

A549 human lung carcinoma cells.

A549 cells (ATCC CCL 185) are grown in high

glucose Dulbecco's modified Eagle's medium (Sigma

D-6546) supplemented with 10% foetal calf serum (PAA),

2 mM glutamine (Sigma G-7513), penicillin/streptomycin

(Sigma P-0781), MEM non-essential amino acids (Sigma

5   M-7145).

Cells are seeded at 1-2 x 105 cells / ml / well

into the wells of 24-well cluster plates and grown

for 24 h in serum-free DMEM. Cell monlayers are

washed twice with serum-free DMEM and 0.5 ml binding

10   medium (DMEM/Hams F12 (Sigma D-6421) containing 0.1%

(v/v) BSA added to each well.

Aliqouts of ^{125}I-TGF-β1 or -β2 (70-90 TBq/mmol;

Amersham International) at 20 pM are preincubated with

antibody in binding medium at room temperature for 1

15   h. Duplicate samples of 0.5 ml of TGF-β/antibody

mixtures are then added to the cell monlayers and are

incubated at 37°C for 1-2 h. Control wells contain

TGF-β only. Unbound TGF-β is removed by washing 4

times with Hank's balanced salt solution containing

20   0.1% BSA. Cells are solubilised in 0.8 ml 25 mM Tris

HCl pH 7.5, 10 % glycerol, 1 % Triton X-100 at room

temperature for 20 min. The contents of each well are

removed and ^{125}I measured in a gamma counter. The

potency of each scFv or IgG is measured by the

25   concentration of antibody combining sites necessary to

inhibit binding of TGF-β by 50% (IC50; Table 5). Thus

the IC50 values are below 10nM and in some cases below

1nM indicating very potent antibodies.

EXAMPLE 5

Prevention of Scar Formation by Antibodies Against TGF β1 and TGF β2 in the Injured Central Nervous System of the Rat

5   Logan *et al* (1994) Eur.3 Neuroscience 6,355-363 showed in a rat model of CNS injury, the ameliorating effect of a neutralising turkey antiserum directed against TGF β_1 on the deposition of fibrous scar tissue and the formation of a limiting glial
10   membrane that borders the lesion. A study was set up to investigate the effects of neutralising engineered human antibodies directed against both TGF β_1 and TGF β_2 in the same rat model. The derivation of the antibodies used in this study is described in examples
15   1 and 2.

Method

Animals and surgery

20   Groups of five female Sprague-Dawley rats (250g) were anaesthetised with an i.p. injection. The anaesthetised rats had a stereotactically defined lesion made into the right occipital cortex (Logan *et al* 1992 Brain Res. 587, P216-227) and the lateral
25   ventricle was surgically cannulated and exteriorised at the same time (Logan *et al* 1994 supra).

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Animals were intraventricularly injected daily
with 5ul of purified anti TGF β antibodies (Table 3)
diluted in a vehicle of artificial cerebrospinal fluid
as described by Logan et al 1994 supra. Fourteen days

5   post lesion all animals were perfusion fixed and 7mm
polyester wax sections were processed for
histochemical evaluation of the lesion site by
immunofluorescent staining.

10   Fluorescent immunohistochemistry and image analysis
Morphological changes within the wound site were
followed by immunofluorescent staining with antibodies
to fibronectin and laminin detected with anti-species
FITC conjugates (Logan et al 1994 supra). These

15   changes were semi-quantitatively assessed by image
analysis using a Leitz confocal microscope linked to a
Biorad MRC500 laser scanning system. Readings were
taken at standard positions mid-way along the lesion.

20   *Results*

Effects of antibodies to TGF β at the site of CNS
injury
Quantitation of the specific relative

25   fluorescence for each of the antibodies is shown in
figure 12 a and b. Laminin is a measure of the
formation of the glial limitans externa along the
boundaries of the wound and together with fibronectin

forms a matrix of fibrous tissue within the centre of

the wound. Quantitation by image analysis of these

two proteins allows the degree of scarring at the

wound site to be determined.

5   Compared with the saline control (fig.12 a,b),

There is a considerable decrease in fibronectin and

laminin immuno-localisation in the wound in the

anti-TGF β antibody treated brains. Thus this

indicates that these engineered human antibodies

10   directed against epitopes on TGF β_1 & TGF β_2 ameliorate

the effects of injury to the CNS both separately and

together. by preventing the deposition of the

cellular matrix proteins fibronectin and laminin

within the wound site. Previously Logan *et al* (1994

15   supra) had shown the effectiveness of a polyclonal

turkey anti-sera directed against TGF β_1. This is the

first report of any antibodies directed against TGF

β_2 having been shown to be effective in this model.

20   *EXAMPLE 6*

Determination of Binding of 6B1 IgG4 to Active or

Latent Form of TGF β_2

        TGFβ_2 is synthesised and secreted exclusively as

a biologically inactive or latent complex (Pircher et

25   al, (1986) Biochem. Biophys Res. Commun. <u>158</u>, 30-37).

The latent complex consists of TGFβ_2 disulphide linked

homodimer non-covalently associated with latency-

associated peptide (LAP). Activation of TGFβ_2 occurs

96

when it is released from it processed precursor.
Active TGFβ$_2$ is capable of reversibly dissociating and
reassociating with the LAP, which results in the
turning on and off of its bio-activity respectively.

5   Cultured PC-3 adenocarcinoma cells (Ikeda *et al*
(1987) Biochemistry <u>26</u>, 2406-2410) have been shown to
secrete almost exclusively latent TGFβ$_2$ providing a
convenient source for determination of binding to the
active or latent form of TGFβ$_2$ by the antibody 6B1

10   IgG4.

Method

Cell Culture

15   PC-3 prostatic adenocarcinoma cells were grown to
confluence in supplemented with 10% FBS. The cells
were washed 3x with PBS and cells cultured for a
further 7 days in serum free Hams F12/DMEM
supplemented with 1.4 x 10^{-5}M tamoxifen (Brown *et al*,

20   (1990) Growth Factors <u>3</u>, 35-43). The medium was
removed, clarified by centrifugation and divided into
two 15ml aliquots. One aliquot was acidified for 15
min with 5M HCl by adding dropwise until the pH = 3.5
and then neutralised by the similar addition of 5M

25   NaOH/1M HEPES pH7.4. This procedure activates the
latent TGFβ2 quantitatively.

Competition ELISA

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Sixteen wells of an ELISA plate were coated
overnight with 100μl 200ng/ml TGFβ$_2$ in PBS at 4°C.
The plate was washed 3x with PBS tween and blocked at
37°C with 200μl of 3% Marvel in PBS.

5   The following samples were incubated at room
temperature for 1 hour.

400μl Hams F12/DMEM (reagent blank)

400μl Hams F12/DMEM plus 4μg 6B1 IgG4 antibody
(positive control)

10   400μl PC 3 acid activated conditioned media plus
4μg 6B1 IgG4 antibody (active TGFβ$_2$ sample)

400μl PC 3 untreated conditioned media plus 4μg
6B1 IgG4 antibody (latent TGFβ$_2$ sample)

The ELISA plate was emptied of blocking solution
15   and 100μl of one of the above solutions added to
sensitised wells in quadruplicate and incubated at
room temperature for 2 hours. The plate was washed 3x
with PBS/Tween and wells refilled with 100μl of goat
anti-human IgG γ chain alkaline phosphatase conjugate
20   diluted 1:5000 in 1% Marvel/PBS. After 1 hour the
wells were washed 3x with PBS/Tween and bound antibody
was revealed with *p*-NPP substrate by absorbance at 405
nm.

25   *Results*

The results of this experiment are shown in
Figure 23.

This result clearly shows that pre-incubation

with activated TGFβ2 inhibits binding of 6B1 to TGFβ2
bound onto an ELISA plate, whereas the latent form
does not. This proves that 6B1 IgG4 only binds to the
active form of TGFβ2.

5

EXAMPLE 7

*Neutralisation by antibodies directed against TGFβ2 of
the inhibitory effect of TGFβ isoforms on cell
proliferation*

10   The neutralising activity of 6B1 IgG4, 6H1 IgG4
(purified as in example 2) and a mouse monoclonal
antibody (Genzyme; J.R. Dasch et al., supra) was
measured for each of the TGFβ isoforms, TGFβ1, TGFβ2
and TGFβ3 in the TF1 cell proliferation assay
15   described in Example 3. The concentration of TGFβ
isoform was 100pM in each assay.

     As shown in Figure 16, 6B1 IgG4 strongly
neutralises TGFβ2 with an IC_{50} of approximately 2nM
(Table 6). This compares to 10nM for the mouse
20   monoclonal from Genzyme and 12nM for 6H1 IgG4.
Neither 6B1 IgG4 nor 6H1 IgG4 significantly neutralise
TGFβ1 (Fig. 17). However, there is significant
neutralisation of TGFβ3 by both 6B1 (IC_{50} ca. 11nM) and
6H1 IgG4 ca. 20nM; Fig. 18). This is considerably
25   less than the neutralisation potency of the Genzyme
monoclonal (IC_{50} ca. 0.1nM).

     Both 6B1 IgG4 and 6H1 IgG4 are stronger
neutralisers of TGFβ2 activity than of TGFgβ3

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activity. The neutralisation of TGFβ3 activity is
greater than would be predicted from the relative
binding of these two isoforms by the antibodies
(example 2) and the relative binding in a

5   radioreceptor assay (example 8).

EXAMPLE 8

Inhibition by antibodies directed against TGFβ2 of
binding of other TGFβ isoforms to receptors measured

10   *in a radioreceptor assay*

        The ability of 6B1 IgG4 to inhibit binding of
TGFβ isoforms to receptors was measured in a
radioreceptor assay as described in example 4.

        6B1 IgG4 inhibited binding of ^{125}I-TGFβ2 with an

15   IC_{50} of 0.05nM. There was no significant inhibition of
binding of ^{125}I-TGFβ1 whereas for ^{125}I-TGFβ3 6B1 IgG4
inhibited binding with an IC_{50} of approximately 4nM
(Table 6). This indicates the potency of 6B1 IgG4 in
this assay and its selectivity for the neutralisation

20   of TGFβ2 activity. Cross-reactivity with TGFβ3 in
this assay is less than 2%.

        Thus 6B1 IgG4 preferentially inhibits the binding
of TGFβ2 to its receptors compared with binding of
TGFβ3.

25

EXAMPLE 9

Assessment of TGFβ1 Antibodies for Therapeutic Use

        The antibodies isolated in Example 1 were

assessed for potential therapeutic value by *in vitro*

measurements of the ability to inhibit TGFβ1 binding

to its receptors and *in vitro* binding properties.

In Example 4 (Table 5) CS32 showed the strongest

5   inhibition of the antibodies tested of the binding of

^{125}I-TGFβ1 to receptors on A549 cells. A further

comparison was performed between CS32 and further

antibodies (CS35, CS37 and CS38) that were isolated as

described in the experiment in Example 1, section 5c.

10   This showed that CS37 appeared to be the most potent

of these antibodies in this assay with an IC$_{50}$ of

approximately 8nM, compared with 40nM for CS32. The

IC50 value for CS32 is higher than in the previous

assay (Table 5) because the nature of the assay means

15   that the absolute IC$_{50}$ value can vary with assay

conditions.

The antibodies 1A-E5 and 1AH-6 (Example1, section

1f) and antibodies derived from them were much less

potent than antibodies derived from 1B2 in

20   neutralising TGFβ activity in this radioreceptor

assay.

Thus CS37 was the most potent antibody candidate

as assessed by inhibition of binding of ^{125}I-TGFβ1 to

its receptor.

25

Assessment of binding to TGFβ3 by anti-TGFβ1

antibodies

The antibodies 14A1 and 10A6 (Example 1, section

2 (a) (iii)) were shown to preferentially bind TGFβ1

over TGFβ2 and TGFβ3 using the same specificity ELISA

as was described in Example 1, section 1 (d) (iii),

except that microtitre plates were coated with 50μl of

5   either 0.2μg/ml TGFβ1; 0.2μg/ml TGFβ2; 0.2 μg/ml

TGFβ3; 10μg/ml bovine serum albumin (BSA) or PBS (the

uncoated well). The clones were shown to be specific

for TGFβ1 since the signal generated in the TGFβ1

coated well was at least five fold greater than the

10   signal on TGFβ2 and TGFβ3.

Antibodies derived from the same 1B2 lineage as

these antibodies, such as 27C1/10A6 IgG4 (which

contains the same VL as 10A6 and the 27C1 VH was

prepared by mutagenesis of CDR3 residues) should have

15   the same cross-reactivity against TGFβ3.

EXAMPLE 10

Construction of a High Expressing Cell Line for 6B1

IgG4 using the Glutamine Synthase Selection Systemand

20   *Assessment in a Neutralisation Assay*

Construction of p6H1 VH gamma4

6B1 VH was amplified from 6H1 pG4D100 (Example 2)

by PCR using oligonucleotides P16 and P17. This DNA

25   was joined by PCR with a 158bp DNA fragment from

M13VHPCR1 (R. Orlandi et al Proc. Natl. Acad. Sci. USA

86 3833-3837, 1989) containing a signal sequence,

splice sites and an intron, using oligonucleotides P10

and P17. The PCR product was cut with HindIII ad ApaI and cloned into HindIII-ApaI cut pGamma4 (Lonza Biologics plc). A plasmid with the correct insertion was identified and designated p6H1 VH gamma4 (see

5   Figure 20). The VH gene and flanking regions were sequenced at this stage.

Construction of 6B1ΔBam pLN10

The VL gene of 6B1 was amplified from the clone

10   of 6B1 scFv in pCANTAB6 (Example 1) and subcloned into pUC119. The VL gene was then mutated by in vitro mutagenesis to remove an internal BamHI site, modifying the DNA sequence but not the protein sequence. In vitro mutagenesis was performed using

15   the oligonucleotide LamDeltaBamHI (Table 1) using a kit from Amersham International plc. The mutated VL gene was amplified using the primers Vλ3backEuApa and HuJλ2-3ForEuBam and subcloned as an ApaLI-BamHI fragment into the vector vlcassetteCAT1. The VL gene

20   was then cloned as a HindIII-BamHI fragment into the vector pLN10 (Figure 8) to generate the vector 6B1ΔBam pLN10.

Construction of p6B1λ

25   The 6B1 Vλ gene was amplified by PCR from p6B1ΔBampLN10 using oligonucleotides P22 and P26. The Cλ gene was amplified by PCR from pLN10-10A6 (Example 2) using oligonucleotides P25 and P19. The 2 DNAs

were joined by overlapping PCR using the

oligonucleotides P22 and P19 and the product cut with

BstBI and EcoRI and cloned into BstBI-EcoRI cut

pMR15.1 (Lonza Biologics plc). A plasmid with the

5　correct insertion was identified and designated p6B1λ

(Figure 21).

Construction of final expression vector p6B1gamma4gs

p6H1 VHgamma4 and p6B1λ were digested with BamHI

10　and NotI, fragments were purified and ligated

together. A plasmid of the desired configuration was

identified from transformants and designated

p6B1gamma4gs (Figure 22).

Transfection of NS0 with p6B1 gamma4gs

15

Stable transfectants secreting 6B1 IgG4 were

selected by introducing into NS0 myeloma cells p6B1

which includes the glutamine synthetase (gs) gene

which allows growth in glutamine-free (G-) medium

20　(C.R. Bebbington et al Bio/Technology $\underline{10}$ 169-175,

1992). 40μg p6B1 gamma4gs were linearised by

digestion with PvuI. The DNA was electroporated into

1.5×10^7 NS0 cells. Cells were then added to

G+DMEM/10% FCS and 50μl aliquots distributed into 6 x

25　96-well plates and allowed to recover for 24h. The

medium was then made selective by the addition of

150μl G-DMEM/10%FCS. Three weeks later gs⁺

transfectants were screened by ELISA for the ability

to secrete human IgG4λ antibody. The highest
producers were expanded and further analysed. From
this analysis 5D8 was selected as the candidate
production cell line. 5D8 was cloned once by limiting

5   dilution to give the cell line 5D8-2A6.

*Assessment of 6B1 IgG4 derived from cell line 5D8-2A6
in the TF1 neutralisation assay*

6B1 IgG4 was purified from the GS/NS0 cell line

10   5D8-2A6 grown in serum-free medium as described in
Example 2. The 6B1 IgG4 antibody was assayed in the
TF1 neutralisation assay as described in Example 3.
An IC_{50} value of 1.8nM was obtained in this assay.
Subsequent assays of preparations of 6B1 IgG4 derived

15   from the 5D8-2A6 cell line have indicated values of
IC_{50} in the range of 0.65 to 2nM. These are comparable
to the values obtained for 6B1 IgG4 produced from CHO
cells (Example 2) and compare favourably with that
obtained for 6H1 IgG4 derived from a CHO cell line

20   (IC_{50} of 15nM). The values obtained for the IC_{50} for
6B1 IgG4 and 6H1 IgG4 in this example are more
reliable than those obtained in Example 3 and are
shown in Table 4, because of improvements in the assay
and in the expression and purification of the

25   antibodies. The IC_{50} value may however be expected to
vary with the precise conditions of the assay.

Thus the 6B1 IgG4 provides potent neutralisation
of TGFβ2 and is suitable for use as a therapeutic.

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EXAMPLE 11

Determination of the Epitope on TGFβ2 for the Antibody
6B1 using a Peptide Phage Display Library

5   The antibody 6B1 was further characterised by

epitope mapping. This was done by using a peptide

phage display library to select peptide sequences that

bind specifically to 6B1. These peptide sequences

were then compared to the amino acid sequence of

10   TGFβ2. Correlation between peptide sequences that

bind to 6B1 and matching parts of the TGFβ2 amino acid

sequence indicate an epitope of TGFβ2 to which 6B1

binds. An "epitope" is that part of the surface of an

antigen to which a specific antibody binds.

15   In this example, the peptide library used was

constructed as described by Fisch et al (I. Fisch et

al (1996) Proc. Natl. Acad. Sci USA $\underline{93}$ 7761-7766) to

give a phage display library of 1×10^{13} independent

clones. Phage displaying peptides that bind to the

20   antibody 6B1 were selected from this library by

panning. This was performed as described in Example

1.

Purified 6B1 IgG4 antibody at 10μg/ml in 4ml of

PBS was coated onto a plastic tube (Nunc; maxisorp) by

25   incubating overnight at 4˚C. After washing and

blocking with MPBS (see Example 1) an aliquot of the

peptide library containing 5×10^{13} phage in 4ml 3%MPBS

was added to the tube and incubated at room

temperature for 1.5 hours. The tube was washed 10

times with PBST(0.1%), then 10 times with PBS. Bound

phage particles were eluted from the tube by adding

4ml of 100mM triethylamine and incubating the tube

5   stationary for 10 minutes at room temperature. The

eluted phage were then added to a tube containing 2ml

1M-Tris.HCl (pH7.4) and 10ml 2YT broth. The phage

were then added to 20ml of logarithmically growing E.

coli TG1 cells and grown for 1 hour shaking at 100rpm

10   at 37°C. The infected cells were then plated on 2YT

agar medium with 15μg/ml tetracycline in 243mm x 243mm

dishes (Nunc). Plates were incubated at 30°C for 18

hours. Colonies were scraped off the plates into 10

ml 2TY broth containing 15% (v/v) glycerol for storage

15   at -70°C.

250μl of cells from the first round of selection

was used to inoculate 500ml 2YT broth (containing

15μg/ml tetracycline) in a 2 litre conical flask and

grown overnight, at 30°C with shaking at 280rpm. A

20   2ml aliquot of this culture was then taken and

centrifuged to remove all cells. 1ml of this phage

supernatant was the used for a second round of

selection as described above. The pattern of phage

growth and panning was repeated over a third and a

25   fourth round of selection.

Individual colonies from the fourth round of

selection were used to inoculate 100μl 2YT broth

(containing 15μg/ml tetracycline) into individual

wells of 96 well tissue culture plates and grown

overnight with gentle shaking at 100rpm at 30°C.

Glycerol was added to a final concentration of 15%

(v/v) and these master plates were stored frozen at

5   -70°C.

These clones were screened for clones that bound

specifically to the antibody 6B1 in ELISA. Cells from

the master plates were used to inoculate 96 well

tissue culture plates containing 100μl 2YT broth

10   (containing 15μg/ml tetracycline) per well and grown

overnight with gentle shaking at 100rpm at 30°C. The

plates were then centrifuged at 2000rpm. The 100μl

phage supernatants from each well were recovered and

each was mixed with 100μl of 4% skimmed milk powder in

15   2x PBS. 100μl of each of these was then assayed by

phage ELISA. Purified 6B1 IgG4 antibody at 10μg/ml in

PBS was coated onto flexible microtitre plates by

incubating overnight at 4°C. Control plates coated

with an irrelevant IgG4 antibody at 10μg/ml were also

20   prepared. The ELISAs were performed as described in

Example 1, and visualised with the chromagenic

substrate pNPP (Sigma).

Approximately 20% of all the clones analysed

bound to the 6B1 coated plate. None of the clones

25   analysed bound to ELISA plates coated with the

irrelevant antibody. Binding therefore appeared to be

specific for the binding site of the antibody 6B1.

Clones which bound 6B1 were analysed by DNA

sequencing as described by Fisch et al. A total of 31

different clones were sequenced. These were analysed

for possible matches with the sequence of TGFβ2 using

Mac vector software. Of these clones, 12 showed poor

5   matching with the sequence of TGFβ2 and 10 had no

similarity at all. However, there were 4 different

clones (some of which had been selected more than

once) which showed a reasonable match to a region of

the TGFβ2 sequence between amino acid positions 56 to

10   69. Table 8 shows the amino acid sequence of the exon

of each of these clones that appears to be responsible

for binding to 6B1.

None of these clones exactly match the sequence

of TGFβ2 nor is there a single clear consensus

15   sequence between the peptide clones. Nevertheless,

careful examination of the sequences reveals a match

with residues 60 to 64 of TGFβ2 (Table 8). Lining up

four clones with L at position 64 reveals 2 clones

with R at position 60, 1 clone with V at position 61,

20   2 with L at position 62 and 3 with S at position 63.

This provides the sequence RVLSL corresponding to

residues 60 to 64 which form part of the alpha helix

which forms the heel region of TGFβ2. An antibody

recognising this structure would not be expected to

25   make contact with every amino acid residue in the

helix and so a peptide mimicking this sequence could

have considerable sequence variation at positions that

correspond to parts of the helix that do not make

contact. The alpha helix recognised is believed to

form part of the receptor binding region of TGFβ2

(D.L. Griffith et al.(1996) Proc. Natl. Acad. Sci. USA

<u>93</u> 878-883).

5

EXAMPLE 12

Determination by Immunohistochemistry of Binding of

6B1 IgG4 to TGFβ2 in Mammalian Tissue and Absence of

Cross Reactivity

10   To detect TGFβ2 in formalin-fixed tissue sections

that express the cytokine, the tissue section is

generally treated with a protease, pronase E. This

digestion step. unmasks the antigen, possibly

activating latent TGF β2 to give active TGF β2. 6B1

15   IgG4 detects only the active form of TGF β2 (Example

6).

        Using 6B1 IgG4 and immunohistochemical methods

the distribution of TGF β2 was determined in formalin

fixed-paraffin wax embedded rat normal rat kidney, and

20   experimentally lesioned rat brain tissue, following

pronase E digestion.

        The reactivity of 6B1 IgG4 in frozen cryostat

sections of acetone post-fixed normal human tissue was

also ascertained to determine whether there was any

25   binding to other antigens in these tissues.

Method

Rat Tissue

Paraffin embedded rat tissues were de-waxed and
rehydrated through an alcohol series. The sections
were then treated with 0.1% pronase E for exactly 8
5 min and then washed in water. TGF β2 was detected in
the sections using 6B1 IgG4 at 500ng/ml following the
protocol provided with a Vectastain ABC (avidin-
biotin-complex) kit from Vector Laboratories. On
kidney sections, bound antibody was located with
10 alkaline phosphatase and peroxidase was used on rat
brain tissues.

Human Tissue

The following human tissue samples were used:
15 Adrenal, Aorta, Blood, Large intestine, Small
intestine, Cerebrum, Kidney, Lymph Node, Liver, Lung,
Spleen, Pancreas, Skeletal muscle, Cardiac Muscle,
Thyroid, Nerve, Skin, Eye.

Cryostat sections and smears were fixed for 15
20 minutes in acetone before application of 6B1 IgG4
antibody labelled with FITC using Sigma Immunoprobe
kit. The labelled antibody was incubated for 18hr at
4°C, then detected using an indirect alkaline
phosphatase method (detection with anti-FITC antibody
25 followed with anti-species enzyme conjugated
antibody). In instances where endogenous alkaline
phosphatase activity could not be suppressed a
peroxidase detection method was used. No pronase

111

digestion was used in this case, therefore this

procedure would detect only antigens with which the

antibody cross-reacts.

5   *Results*

Rat Tissue

Rat kidneys displayed positive staining in

tubules present on both the apical and the basolateral

10   side, demonstrating the presence of TGF β2 in the

tissues.

Injured rat brain at 5 days post injury showed

positive staining of neurones, astrocytes and

macrophages which was absent in normal brain. This

15   indicates that the TGF β2 is expressed in rat brain

following injury.

Human Tissue

No specific staining of any tissue was observed

20   using fixed cryostat sections of the tissues listed

above. Therefore 6B1 IgG4 does not cross-react with

antigens in these tissues and when used

therapeutically will bind only active TGF β2 in tissue

sections detected by immunohistochemical methods.

25

EXAMPLE 13

Kinetic analysis of the binding of 6B1 single chain Fv

and 6B1 IgG4 to TGFβ isoforms

112

Surface plasmon resonance (SPR) can be used to examine real-time interactions between an immobilised ligand and an analyte, and derive kinetic constants from this data. This was performed using the BIAcore

5   2000 system (Pharmacia Biosensor) with the antigen immobilised on a surface, and the antibody as analyte.

The system utilises the optical properties of surface plasmon resonance to detect alterations in protein concentration within a dextran matrix.

10 Antigen is covalently bound to the dextran matrix at a set amount, and as solution containing antibody passes over the surface to which this is attached, antibody binds to the antigen, and there is a detectable change in the local protein concentration, and therefore an

15 increase in the SPR signal. When the surface is washed with buffer, antibody dissociates from the antigen and there is then a reduction in the SPR signal, so the rate of association, and dissociation, and the amount of antibody bound to the antigen at a

20 given time can all be measured. The changes in SPR signal are recorded as resonance units (RU), and are displayed with respect to time along the y-axis of a sensorgram.

The density of immobilised ligand on the surface

25 of a BIACore chip is important when deriving kinetic data from the sensorgrams generated. It needs to be quite low, so that only a small amount of analyte antibody is needed for saturation of the chip surface.

For simplicity, the density of a chip surface is
quoted in RU's, and an ideal amount for a ligand such
as TGFβ2 or TGFβ3 (25kDa) is 400-600 RU's relative to
the baseline set during the immobilisation of the
5   ligand to the surface. The actual amount of TGFβ that
has to be added to get the correct density has to be
determined by investigation, but is reproducible once
the correct concentration has been found.

Immobilisation of the ligand to the dextran
10   matrix of the chip surface is facilitated via amine
groups, on lysine side chains in the protein, and
carboxyl groups in the dextran matrix. The carboxyl
groups in the dextran are activated with N-
hydroxysuccinimide (NHS) and N-ethyl-N'-(3-
15   diethylaminopropyl) carbodiimide (EDC) the antigen in
acidic solution is then bound to the surface, and
finally any unreacted carboxyl groups are blocked with
ethanolamine.

The immobilisation of ligand is automated by the
20   BIACore 2000 machine, and all steps are carried out in
the autosampler or in the flowcell, on the dextran
surface of the chip. The buffer used throughout the
immobilisation procedure, and the analysis of samples
is Hepes -buffered saline (HBS) with a surfactant
25   (Pharmacia Biosensor). The chips (Pharmacia, CM5),
have dextran coating on a thin layer of gold. NHS at
100mM and EDC at 400mM are mixed by the autosampler,
and then a fixed volume is injected over the flowcell

surface. This is followed by an injection of antigen

in a suitable buffer. In the case of TGFβ, a surface

of the correct density was given by using 25-30μg/ml

solution of TGFβ2 (AMS) OR TGFβ3 (R & D systems) in

5   10mM acetate. After injection of the ligand, the chip

is blocked using 1M ethanolamine. The total amount of

TGFβ bound was assessed from the total increase in

resonance units over this period.

To determine the kinetic parameters, a series of

10 dilutions of the antibody samples was made in HBS from

about 500μg/ml down to less than 1 μg/ml, usually

through doubling dilutions. After the antibody has

been injected over the antigen surface, the surface is

washed with HBS, then regenerated by stripping off the

15 bound antibody with a pulse of 100mM HCl. At the

higher concentrations of antibody the antigen on the

chip surface is saturated, and the off rate is

determined on washing with buffer in the dissociation

phase. For determination of the on-rate, lower

20 concentrations of antibody are used, giving a linear

binding phase in the sensorgram, allowing k_{on}

determination.

The set of dilutions were repeated on a separate

preparation of the same antibody.

25   To manipulate the sensorgrams to obtain kinetic

constants k_{on} and k_{off}, the BIAevaluation software

package is used. For each binding curve used in the

calculations, care was taken that the conditions were

appropriate for the determination of kinetic
constants.

6B1 IgG4 was purified from the GS/NS0 cell line
of Example 10 as in Example 2. 6B1 single chain Fv
5   was expressed intracellularly in *E. coli*, refolded *in
vitro* (using the methodology of WO94/18227), and
purified to give a homogeneous product. The values of
k_{on} and k_{off} were determined for 6B1 IgG4 for binding
to both TGFβ2 and TGFβ3, and for the single-chain Fv
10   6B1 for binding to TGFβ2. The dissociation constant
was calculated by dividing k_{off} by k_{on}. The values for
these kinetic parameters are shown in Table 7.

Thus, 6B1 scFv and 6B1 IgG4 show very low
dissociation constants of 2.3nM and 0.89nM
15   respectively for TGFβ2, and there is 9% cross-
reactivity with TGFβ3 (as judged by the ratio of
dissociation constants of 6B1 IgG4 for TGFβ3 and
TGFβ2). For comparison, in earlier studies, where the
standard errors were greater and the values less
20   precise, the Kd values for TGFβ2 were determined to be
0.7nM for 6A5 scFv (Table 2) and 2nM for 6H1 IgG4
(Example 2). The Kd values for all the antibodies
directed against TGFβ2 which share the same 6H1 VH
domain are low and below 10nM.

25

EXAMPLE 14

*Binding of a Peptide Corresponding to Residues 56 to
69 of TGFβ2 to 6B1 IgG4*

9/2/2005, EAST Version: 2.0.1.4

A peptide was synthesised corresponding to the amino acids of TGFβ2 surrounding the residues RVLSL, the epitope identified from the selection of phage from the peptide display library (Example 11).

5   The 17-mer peptide CGG-TQHSRVLSLYNTIN (TGFβ2$_{56-69}$; synthesised by Cambridge Research Biochemicals) contains residues 56 to 69 of TGFβ2 with RVLSL (residues 60 to 64) at its centre. The CGG N-terminal extension is a spacer with a cysteine residue to

10   facilitate coupling of the peptide to carrier proteins. The peptide corresponding to residues 56 to 69 from TGFβ1 (TGFβ1$_{56-69}$; CGG-TQYSKVLSLYNQHN) was also synthesised. As a control, irrelevant peptide GPEASRPPKLHPG was used.

15   Two approaches were used to confirm that the epitope on TGFβ2 for 6B1 IgG4 comprised the amino acids RVLSL.

(i) Assessment of the ability of 6B1 IgG4 to bind to TGFβ2$_{56-69}$ and TGFβ1$_{56-69}$ coupled to BSA by ELISA

20   (ii) Assessment of the ability of peptides to bind to 6B1 IgG4 coated onto a BIACore sensor chip.

(i) Assessment of the ability of 6B1 IgG4 to bind to TGFβ2$_{56-69}$ and TGFβ1$_{56-69}$ coupled to BSA by ELISA

25   The binding of 6B1 IgG4 to synthetic peptides TGFβ1$_{56-69}$ and TGFβ2$_{56-69}$ conjugated to BSA was assessed in an ELISA assay. This was compared with the binding of a control antibody 2G6 IgG4 which is an engineered

antibody with a heavy chain containing a VH from an

antibody directed against the hapten NIP combined with

a light chain containing a VL from an antibody

directed against lysozyme.

5

Method

Two mg of each of the peptides TGFβ1$_{56-69}$ and

TGFβ2$_{56-69}$ were conjugated to BSA using an Imject

Activated Immunogen Conjugation kit (Pierce).

10   An immunosorp microtitre plate (Nunc) was coated

overnight with 10ug/ml of the conjugated peptides in

PBS   (rows A-D TGFβ1$_{56-69}$, rows E-F TGFβ2$_{56-69}$) at

100μl/well.   The wells were washed 3x with PBS-tween

and the following additions made: Column 1 -100μl PBS

15   in each well as reagent control; Column 2, rows A,B,E

and F 200μl of 6B1 IgG4 10μg/ml; Column 2, rows C,D,G

and H 200μl of 2G6 IgG4 10μg/ml.

100μl of PBS was put into all the remaining

wells.   To produce doubling dilutions of the

20   antibodies, 100μl was removed from each well in column

2 and placed into the next well in column 3.   The

sample was mixed and 100μl removed and added to the

next well in column 4.   This procedure was repeated

along the plate with the last 100μl being discarded.

25   The plate was then incubated at 4°C for 18hr.

After 3x washes with PBS-tween the wells were

refilled with 100ul of an alkaline phosphatase

conjugate of goat F(ab')$_2$ fragment specific for the

human IgG gamma chain diluted 1:1000 in PBS and incubated for a further 1hr. After 3x further washes with PBS-tween bound antibody was revealed with p-NPP substrate for 20min.

5

Results

6B1 IgG4 was shown to bind to both conjugated peptides (Figure 15) but the ELISA signal obtained with $TGF\beta1_{56-69}$ was much lower than that obtained with

10    $TGF\beta2_{56-69}$ at an equivalent concentration of 6B1 IgG4. An approximately 8 to 10 times higher concentration of 6B1 IgG4 was required to obtain an equivalent signal with $TGF\beta1_{56-69}$ compared with $TGF\beta2_{56-69}$. No signal was obtained with the control 2G6 IgG4 antibody with

15   either peptide-BSA conjugate. 6B1 IgG4 therefore strongly binds TGFb256-69 and more weakly binds $TGF\beta1_{56-69}$ coupled to BSA.

(ii) Assessment of the ability of peptides to bind to

20   *6B1 IgG4 coated onto a BIACore sensor chip.*

The binding of 6B1 IgG4 to $TGF\beta2_{56-69}$ was confirmed by binding the peptide to 6B1 IgG4 coated on to a BIACore sensor chip. The determination of binding properties by surface plasmon resonance using

25   the Pharmacia BIACore 2000 was described in Example 13. The method of creating a BIACore sensor chip coated with 6B1 IgG4 was as for the method for coupling with $TGF\beta$, described in Example 13, except

that 6B1 IgG4 was coupled at 5µg/ml in 10mM acetate

buffer, pH3.5. A surface of 5000RU was generated

using 25µl of 6B1 IgG4.

Twenty µl of the the peptides were applied to the

5   6B1 surface at 1mg/ml with regeneration of the surface

using an acid pulse to remove bound peptide between

samples. The amount of binding was assessed by

setting a baseline response of absolute RU prior to

injection, and then subtracting this from the value at

10   20 seconds after the injection was complete to give a

relative response in RU. This is taken to be the

amount of binding to the 6B1 surface.

The binding obtained is shown in Table 9. There

was a very low level of binding of the irrelevant

15   peptide. TGF$\beta 1_{56-69}$ appeared to bind specifically at a

low level to 6B1 IgG4. However, the TGF$\beta 2_{56-69}$ peptide

bound to 6B1 IgG4 specifically and very much more

strongly.

The low level of binding of 6B1 IgG4 to the TGFβ1

20   peptide in the ELISA and BIACore assays is not

unexpected given that 10 of the 14 TGFβ amino acids

are identical with the TGFβ2 peptide. Nevertheless,

6B1 IgG4 binds the TGF$\beta 2_{56-69}$ peptide very much more

strongly than it binds the TGF$\beta 1_{56-69}$ peptide. The

25   level of discrimination between these TGFβ1 and TGFβ2

peptides is very much lower however than is seen for

the radioreceptor (Table 6) and neutralisation assays

(Table 6 and Figures 16 and 17) with native isoforms.

In these assays, 6B1 IgG4 strongly neutralises TGFβ2
but has little effect on TGFβ1 biological activity.
This greater discrimination presumably reflects the
context of the residues of the peptides in the native
5   isoforms.

Conclusions

       These results support the assignment of the
epitope of 6B1 IgG4 on TGFβ2 to the aminoacids in the
10   region of residues 60 to 64. The peptide used in this
example, residues 56 to 69, corresponds to the amino
acids of alpha helix H3 (M.P. Schlunegger & M.G.
Grutter Nature <u>358</u> 430-434, 1992). TGFβ2 forms a
head-to-tail dimer with the alpha helix H3 (also
15   referred to as the heel) of one subunit forming an
interface with finger regions (including residues 24
to 37 and residues in the region of amino acids 91 to
95; also referred to as fingers 1 and 2) from the
other subunit (S. Daopin et al Proteins: Structure,
20   Function and Genetics <u>17</u> 176-192, 1993). It has been
proposed that the primary structural features which
interact with the TGFβ2 receptor consist of amino
acids at the C-terminal end of the alpha helix H3 from
one chain together with residues of fingers 1 and 2 of
25   the other chain (D.L. Griffith et al Proc. Natl. Acad.
Sci. USA <u>93</u> 878-883, 1996). The identification of an
epitope for 6B1 IgG4 within the alpha helix H3 of
TGFβ2 is consistent with 6B1 IgG4 preventing receptor

binding and neutralising the biological activity of
TGFβ2.

If the epitope for 6B1 IgG4 is three dimensional
there may be other non-contiguous epitopes to which

5   the antibody may bind.

There is earlier evidence that antibodies
directed against this region of TGFβ2 may be specific
for TGFβ2 and neutralise its activity. Flanders et al
(Development 113 183-191 1991) showed that polyclonal

10   antisera could be raised in rabbits against residues
50 to 75 of mature TGFβ2 and that these antibodies
recognised TGFβ2 but not TGFβ1 in Western blots. In
an earlier paper, K.C. Flanders et al (Biochemistry 27
739-746, 1988) showed that polyclonal antisera raised

15   in rabbits against amino acids 50 to 75 of TGFβ1 could
neutralise the biological activity of TGFβ1. The
antibody we have isolated and characterised, 6B1 IgG4,
is a human antibody directed against amino acids in
this region which neutralises the biological activity

20   of human TGFβ2. It is surprising that such a
neutralising antibody against TGFβ2 can be isolated in
humans (where immunisation with a peptide cannot be
used for ethical reasons) directly from a phage
display antibody repertoire.

Table 1: Oligonucleotide primers used in the identification and characterisation of TGF-b1 antibodies.

Primer	Nucleotide sequence 5' to 3'
iB2 mutVHCDR3	5' CGT GGT CCC TTT GCC CCA GAC GTC CAC ACC ACT AGA ATC GTA GCC ACT ATA TTC CCC AGT TCG CGC ACA GTA ATA CAC AGC CGT
pUC19reverse	5' AGC GGA TAA CAA TTT CAC ACA GG 3'
fdtet seq	5' GTC GTC TTT CCA GAC GTT AGT 3'
PCR-H-Link	5' ACC GCC AGA GCC ACC TCC GCC 3'
PCR-L-Link	5' GGC GGA GGT GGC TCT GGC GGT 3'
myc seq 10	5' CTC TTC TGA GAT GAG TTT TTG 3'
HUJH4-5For	5' TGA GGA GAC GGT GAC CAG GGT TCC 3'
RL1	5' G(C/A)A CCC TGG TCA CCG TCT CCT CA GGT GGA GGC GGT TCA GGC GGA GGT GGC AGC GGC GGT GGC GGA TCG 3'
RL2	5' GGA CAA TGG TCA CCG TCT CTT CA GGT GGA GGC GGT TCA GGC GGA GGT GGC AGC GGC GGT GGC GGA TCG 3'
RL3	5' GGA CCA CGG TCA CCG TCT CCT CA GGT GGA GGC GGT TCA GGC GGA GGT GGC AGC GGC GGT GGC GGA TCG 3'
VH1b/7a back Sfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG (AG)TG CAG CTG GTG CA(AG) TCT GG-3'
VH1c back Sfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC (GC)AG GTC CAG CTG GT(AG) CAG TCT GG-3'

VH2b back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG (AG)TC ACC TTG AAG GAG TCT GG-3'

VH 3b back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC (GC)AG GTG CAG CTG GTG GAG TCT GG-3'

VH3c back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTG GTG GAG (AT)C(TC) GG-3'

VH4b back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTA CAG CAG TGG GG-3'

VH4c back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG (GC)TG CAG CTG CAG GAG TC(GC) GG-3'

VH5b back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GA(AG) GTG CAG CTG GTG CAG TCT GG-3'

VH 6a back Sfi
5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTA CAG CTG CAG CAG TCA GG-3'

VH3BACKSfiEu   5'- AGC TCG GTC CTC GCA ACT GCG GCC CCT GGG GCC CAC AGC GAG GTG CAG CTG GTG GAG TCT GG - 3'

VHJH6FORBam   5'-CGA GTC ATT CTG CAC TTG GAT CCA CTC ACC TGA GGA GAC GGT GAC CGT GGT CCC - 3'

DeltaBamHI   5'-GA GAA TCG GTC TGG GAT TCC TGA GGG CCG G-3'

Vλ3/4BackEuApa   5'- AGC TCG GTC CTC GCA ACT GCG GAT CCA CTC ACC GGT GTG CAC TCC CAC GTT ATA CTG ACT CAG GAC CC -3'

HuJλ2-3ForEuBam   5'-G GTC CTC GCA ACT GCG GAT CCA CTC ACC TAG GAC GGT CAG CTT GGT CCC- 3'

VHJH1-2FORBam   5'-CGA GTC ATT CTG CAC TTG GAT CCA CTC ACC TGA GGA GAC GGT GAC CAG GGT GCC - 3'

Vκ2BackEuApa 5'- AGC TCG GTC CTC GCA ACT GGT GTG CAC TCC GAT GTT GTG ATG ACT CAG TCT CC-3'

HuJκForEuBam 5'-G GTC CTC GCA ACT GCG GAT CCA CTC ACG TTT GAT ATC CAC TTT GGT CCC -3'

Vλ3BackEuApa 5'- AGC TCG GTC CTC GCA ACT GGT GTG CAC TCC TCG TCT GAG CTG ACT CAG GAC CC -3'

LamDeltaBamHI 5'- C CGG CCC TCA GGA ATC CCA GAC CGA TTC TC- 3'

P10   5'-CTA AGC TTA CTG AGC ACA CAG GAC CTC ACC-3'

P16   5'-TTT GGA TAT CTC TCC ACA GTC CAC TCC GAG GTG CAG CTG GTG GAG TCT G-3'

P17   5'-ATG GGC CCT TGG AAG CTG AAG AGA CGG TGA CCA GGG TGC C-3'

P19   5'-TTG AAT TCA GGT GGG GGC ACT TCT CCC TCT ATG AAC ATT CCG TAG GGG CCA CTG TCT TC-3'

P22   5'-TTA ACG ATT TCG AAC GCC ACC ATG GGA TGG AGC TGT ATC ATC CTC-3'

P25   5'-GTC CTA GGT GAG TAG ATC TAT CTG GGA TAA GCA TGC TGT TTT C-3'

P26   5'-GAT CTA CTC ACC TAG GAC GGT CAG CTT GG-3'

Table 2 Properties of single chain Fv fragments for binding to TGFbeta1 or
TGFbeta2 determined using BIACore

Antibody	k_{off} (s^{-1})	$K_d(nM)$
TGFbeta1		
31G9	9.0×10^{-4}	12
CS32	1.2×10^{-3}	
CS39	1.7×10^{-3}	
TGFbeta2		
6A5	1.4×10^{-4}	0.7
6B1	6.0×10^{-4}	
6H1	1.1×10^{-3}	
14F12	2.1×10^{-3}	

Table 3 Daily dose levels for individual animals in each group

Group	Clone	Antibody format	Antigen	Dose
1	Saline Control	-	-	-
2	31G9	scFv	TGF β_1	20ng
3	6A5	scFv	TGF β_2	20ng
4	27C1/10A6	IgG4	TGF β_1	692ng
5	6H1	IgG4	TGF β_2	1.76μg
6	31G9 +6A5	scFv's	TGF β_1 TGF β_2	20ng "
7	27C1/10A6 + 6H1	IgG4's	TGF β_1 TGF β_2	692ng 1.76μg

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Table 4 I.C.$_{50}$ values for antibodies in TF1 assay

Antibody	scFv (nM)	IgG4 (nM)
6H1	1.5	100
6B1	15	11
6A5	8	150
14F12	90	nd

nd = not determined

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Table 5 IC$_{50}$ values for antibodies measured using a radioreceptor assay.

Anti-TGF-β1 antibody	IC$_{50}$, nM
7A3 scFv	>100
31G9 scFv	30
CS32 scFv	4.5
CS39 scFv	~60
27C1/10A6 IgG	9
VT37 scFv	~100

Anti-TGF-β2 antibody	IC$_{50}$, nM
6A5 scFv	1.5
6A5 IgG	~6
6B1 scFv	0.3
6B1 IgG	0.6
6H1 scFv	0.22
6H1 IgG	~10
11E6 IgG	1.6
14F12 scFv	3
VT37 scFv	2

Table 6 Potency of neutralisation of TGFbeta isoforms

TF1 cell proliferation assay IC_{50} (nM IgG)		
	6B1 IgG4	Genzyme
TGFbeta1	>100	1.5
TGFbeta2	2	10
TGFbeta3	11	0.1
A549 cell radioreceptor assay IC_{50} (nM IgG)		
	6B1 IgG4	Genzyme
TGFbeta1	>400	0.55
TGFbeta2	0.05	0.5
TGFbeta3	4	0.03

Table 7   Kinetic parameters of 6B1 IgG4 and 6B1 single chain Fv

antibody format	antigen	k_{off} s^{-1}	k_{on} $M^{-1}s^{-1}$	dissociation constant K_d nM
6B1 scFv	TGFβ2	6.68×10^{-4}	2.87×10^{5}	2.32
6B1 IgG4	TGFβ2	3.36×10^{-4}	3.84×10^{5}	0.89
6B1 IgG4	TGFβ3	4.5×10^{-4}	4.5×10^{4}	10.0

Table 8   Peptide sequences from phage binding to 6B1 IgG4

This table shows the amino acid sequence of 4 phage peptide
display clones that show a match with the sequence of
TGFbeta2. These clones have been lined up below the
relevant part of the sequence of TGFbeta2, which is shown
from amino acid positions 56 to 77.

TGFbeta2   TQHSRVLSLYNTINPEASASPC
Clone 1   RQLSLQQRMH
Clone 2   DPMDMVLKLC
Clone 3   WSEFMRQSSL
Clone 3   VESTSLQFRG

peptide	concentration of peptide, µM	amount of binding to 6B1 IgG4 surface, RU
TGFβ2$_{56-69}$	537	1012.8
TGFβ1$_{56-69}$	524	190.7
irrelevant peptide	745	60.9

Table 9 Binding of peptides from TGFbeta to 6B1 IgG4 immobilised on a BIACore chip

CLAIMS:

1.   A specific binding member comprising a human
antibody antigen binding domain specific for human TGFβ
which binds the human TGFβ isoforms TGFβ2, TGFβ1, or

5   TGFβ2 and TGFβ1, preferentially over TGFβ3.

2.   A specific binding member according to claim 1
which neutralises TGFβ2, TGFβ1, or TGFβ2 and TGFβ1.

3.   A specific binding member according to claim 1 or
claim 2 wherein said human antibody antigen binding

10   domain is for the TGF-β isoform TGF-β2.

4.   A specific binding member according to claim 3
wherein said human antibody antigen binding domain
comprises a VH domain which has the amino acid sequence
shown in Figure 2(a) (i) or Figure 2(a) (ii).

15   5.   A specific binding member according to claim 3 or
claim 4 wherein said human antibody antigen binding
domain comprises a VL domain which has the amino acid
sequence shown in any of Figures 2(b) (i) to (v)

6.   A specific binding member according to claim 5
20   wherein said human antibody antigen binding domain
comprises a pairing of a VH domain and a VL domain
selected from:
(a)   6H1 VH, of which the amino acid sequence is shown

in Figure 2(a) (i), and 6B1 VL, of which the amino acid
sequence is shown in Figure 2(b) (iii);

(b)   6H1 VH, of which the amino acid sequence is shown
in Figure 2(a) (i), and 6H1, of which the amino acid

5   sequence is shown in Figure 2(b) (i);

(c)   6H1 VH, of which the amino acid sequence is shown
in Figure 2(a) (i), and 6A5 VL, of which the amino acid
sequence is shown in Figure 2(b) (ii).

7.   A specific binding member according to claim 6

10 wherein said human antibody antigen binding domain
comprises the VH domain 6H1 VH, of which the amino acid
sequence is shown in Figure 2(a) (i), and the VL domain
6B1 VL, of which the amino acid sequence is shown in
Figure 2(b) (iii).

15 8.   A specific binding member according to claim 3
wherein said human antibody antigen binding domain
comprises a complementarity determining region (CDR)
with an amino acid sequence identified as a CDR in any
of the sequences shown in Figures 19 (i) to (iv).

20 9.   A specific binding member according to claim 8
wherein said human antibody antigen binding domain
comprises a VH domain which comprises a CDR3 with a
sequence shown as CDR3 in Figure 19 (i).

10.   A specific binding member according to claim 3

which competes for binding to TGF-β2 with a specific
binding member according to claim 6.

11.   A specific binding member according to claim 10
which competes for binding to TGF-β2 with a specific
5   binding member according to claim 7.

12.   A specific binding member according to claim 3
which binds the peptide TQHSRVLSLYNTIN.

13.   A specific binding member according to claim 3
which binds the active form of TGFβ2 but not the latent
10   form.

14.   A specific binding member according to claim 3
wherein said human antibody antigen binding domain
comprises a VH sequence of the DP50 germ line, or a re-
arranged form thereof.

15   15.   A specific binding member according to claim 1 or
claim 2 wherein said human antibody antigen binding
domain is for the TGF-β isoform TGF-β1.

16.   A specific binding member according to claim 15
wherein said human antibody antigen binding domain
20   comprises a VH domain which has the amino acid sequence
shown in any of Figure 1(a) (i), Figure 1(a) (ii) and
Figure 1(c) (i).

17.   A specific binding member according to claim 15
or claim 16 wherein said human antibody antigen binding
domain comprises a VL domain which has the amino acid
sequence shown in any of Figures 1(b) (i), 1(b) (ii)
5   and 1(a) (iii).

18.   A specific binding member according to claim 17
wherein said human antibody antigen binding domain
comprises a pairing of a VH domain and a VL domain
selected from:
10   (a)   1B2 VH, of which the amino acid sequence is shown
in Figure 1(a) (i), and 7A3 VL, of which the amino acid
sequence is shown in Figure 1(b) (i);
(b)   31G9 VH, of which the amino acid sequence is
shown in Figure 1(a) (ii), and 31G9 VL, of which the
15   amino acid sequence is shown in Figure 1(a) (iii);
(c)   27C1 VH, of which the amino acid sequence is
shown in Figure 1(c) (i), and 10A6 VL, of which the
amino acid sequence is shown in Figure 1(b) (ii).

19.   A specific binding member according to claim 18
20   wherein said human antibody antigen binding domain
comprises the VH domain 27C1 VH, of which the amino
acid sequence is shown in Figure 1(c) (i), and the VL
domain 10A6 VL, of which the amino acid sequence is
shown in Figure 1(b) (ii).

25   20.   A specific binding member according to claim 15

wherein said human antibody antigen binding domain

comprises a VH domain which comprises a CDR3 with an

amino acid sequence selected from those shown in Figure

3.

5   21.   A specific binding member according to claim 20

wherein said CDR3 has the sequence shown for CDR3 of

27C1 VH.

22.   A specific binding member according to claim 15

wherein said human antibody antigen binding domain is

10   comprises the 31G9 VH domain of which the sequence is

shown in Figure 1(a) (ii) and the CS37 VL of which the

sequence is shown in Figure 14.

23.   A specific binding member according to claim 15

which competes for binding to TGF-β1 with a specific

15   binding member according to claim 18.

24.   A specific binding member according to claim 23

which competes for binding to TGF-β1 with a specific

binding member according to claim 19.

25.   A specific binding member according to claim 15

20   which competes for binding to TGFβ1 with a specific

binding member according to claim 22.

26.   A specific binding member according to claim 15

which binds the peptide TQYSKVLSLYNQHN.

27. A specific binding member according to claim 1 wherein said human antibody antigen binding domain is for the TGF-β isoforms TGF-β1 and TGF-β2.

5 28. A specific binding member according to claim 27 wherein said human antibody antigen binding domain comprise a VL domain with the amino acid sequence shown in Figure 4 and a VH domain with the amino acid sequence shown in Figure 1(a) (ii).

10 29. A specific binding member according to claim 27 which competes for binding to TGF-β1 and for binding to TGF-β2 with a specific binding member according to claim 28.

30. A specific binding member according to any

15 preceding claim comprising a single-chain Fv antibody molecule.

31. A specific binding member according to any of claims 1 to 29 which comprises one or more amino acids in addition to those forming said human antibody

20 antigen binding domain.

32. A specific binding member according to claim 31 comprising an antibody constant region.

33.   A specific binding member according to claim 32
which comprises a whole antibody.

34.   A specific binding member according to claim 32
or 33 wherein said antibody constant region is IgG4
5   isotype.

35.   A method comprising causing or allowing binding
of a specific binding member according to any preceding
claim to TGF-β1 isoform and/or TGF-β2 isoform of human
TGF-β.

10   36.   A method according to claim 35 wherein binding
takes place *in vitro*.

37.   A method according to claim 35 wherein binding
takes place *in vivo*.

38.   A method according to any of claims 35 to 37
15   wherein said binding of the specific binding member
neutralises said isoform or isoforms.

39.   Use of a specific binding member according to any
of claims 1 to 34 in the manufacture of a medicament
for treating an individual to counteract effects of
20   TGF-β which are deleterious to the individual.

40.   Use according to claim 39 wherein said effects

140

are fibrosis promoting effects.

41.   Use according to claim 40 wherein said individual
has a condition selected from the group consisting of
glomerulonephritis, neural scarring, dermal scarring,
5   ocular scarring, lung fibrosis, arterial injury,
proliferative retinopathy, retinal detachment, adult
respiratory distress syndrome, liver cirrhosis, post
myocardial infarction, post angioplasty restenosis,
keloid scarring, scleroderma, vascular disorders,
10   cataract, and glaucoma.

42.   Use according to claim 41 wherein said condition
is neural scarring or glomerulonephritis.

43.   Use according to claim 39 wherein said effects
contribute to an immune or inflammatory disease
15   condition.

44.   Use according to claim 43 wherein said condition
is selected from the group consisting of rheumatoid
arthritis, macrophage deficiency disease and macrophage
pathogen infection.

20   45.   Nucleic acid encoding a specific binding member
according to any of claims 1 to 34.

46.   Nucleic acid according to claim 45 which is part

of an expression vector.

47.   A method which comprises use of nucleic acid
according to claim 45 or claim 46 in an expression
system for production of a specific binding member
5   according to any of claims 1 to 29.

48.   A host cell containing nucleic acid according to
claim 45 or claim 46.

49.   A host cell according to claim 48 which is
capable of producing said specific binding member under
10   appropriate culture conditions.

50.   A method of producing a specific binding member
according to any of claims 1 to 34 comprising culturing
a host cell according to claim 49 under appropriate
conditions for production of said specific binding
15   member.

51.   A method according to claim 50 wherein following
said production said specific binding member is
isolated from the cell culture.

52.   A method according to claim 51 wherein following
20   said isolation the specific binding member is used in
formulation of a composition comprising at least one
additional component.

53.   A method according to claim 52 wherein said
composition is a pharmaceutical composition comprising
a pharmaceutically acceptable excipient.

54.   A pharmaceutical composition comprising a
5   specific binding member according to any of claims 1 to
34 and a pharmaceutically acceptable excipient.

55.   A method of treatment of a condition in which
effects of TGF-β are deleterious to an individual, the
method comprising administration of a pharmaceutical
10   composition according to claim 54 to the individual.

56.   A method according to claim 50 wherein said
effects are fibrosis promoting effects.

57.   A method according to claim 56 wherein said
individual has a condition selected from the group
15   consisting of glomerulonephritis, neural scarring,
dermal scarring, ocular scarring, lung fibrosis,
arterial injury, proliferative retinopathy, retinal
detachment, adult respiratory distress syndrome, liver
cirrhosis, post myocardial infarction, post angioplasty
20   restenosis, keloid scarring, scleroderma, vascular
disorders, cataract, and glaucoma.

58.   A method according to claim 57 wherein said
condition is neural scarring or glomerulonephritis.

59.   A method according to claim 55 wherein said

effects contribute to an immune or inflammatory disease

condition.

60.   A method according to claim 59 wherein said

5   condition is selected from the group consisting of

rheumatoid arthritis, macrophage deficiency disease and

macrophage pathogen infection.

Figure 1(a)(i)

```
                10              20              30          40
CAG GTG CAA CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG
 Q   V   Q   L   V   E   S   G   G   G   V   V   Q   P   G   R>

 50              60              70          80          90
TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT AGC TAT
 S   L   R   L   S   C   A   A   S   G   F   T   F   S   S   Y>

100             110             120             130         140
GGC ATG CAC TGG GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG
 G   M   H   W   V   R   Q   A   P   G   K   G   L   E   W   V>

150             160             170             180         190
GCA GTT ATA TCA TAT GAT GGA AGT AAT AAA TAC TAT GCA GAC TCC GTG
 A   V   I   S   Y   D   G   S   N   K   Y   Y   A   D   S   V>

200             210             220             230         240
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
 K   G   R   F   T   I   S   R   D   N   S   K   N   T   L   Y>

250             260             270             280
CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT
 L   Q   M   N   S   L   R   A   E   D   T   A   V   Y   Y   C>

290             300             310         320         330
GCG AAA ACT GGG GAA TAT AGT GGT TAC GAT GGT TCT AGT GGT GTG GAC GTC
 A   K   T   G   E   Y   S   G   Y   D   S   G   V   D   V>

340             350             360
TGG GGC AAA GGG ACC ACG GTC ACC GTC TCC TCA
 W   G   K   G   T   T   V   T   V   S   S
```

Figure 1(a)(ii)

```
                    10              20              30      40
CAG GTG CAG CTG GTG CAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG
 Q   V   Q   L   V   Q   S   G   G   G   V   V   Q   P   G   R >

50              60              70          80              90
TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT AGC TAT
 S   L   R   L   S   C   A   A   S   G   F   T   F   S   S   Y >

100             110             120             130     140
GGC ATG CAC TGG GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG
 G   M   H   W   V   R   Q   A   P   G   K   G   L   E   W   V >

150             160             170     180         190
GCA GTT ATA TCA TAT GAT GGA AGT ATT AAA TAC TAT GCA GAC TCC GTG
 A   V   I   S   Y   D   G   S   I   K   Y   Y   A   D   S   V >

200             210         220     230         240
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
 K   G   R   F   T   I   S   R   D   N   S   K   N   T   L   Y >

250         260             270         280
CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT
 L   Q   M   N   S   L   R   A   E   D   T   A   V   Y   Y   C >

290             300             310         320         330
GCG CGA ACT GGT GAA TAT GGT AGT GGC TAC GAT AGT ACG GGT GTG GAG CTC
 A   R   T   G   E   Y   G   S   G   Y   D   S   T   G   V   E   L >

340             350             360
TGG GGG CAA GGG ACC ACG GTC ACC GTC TCC TCA
 W   G   Q   G   T   T   V   T   V   S   S
```

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Figure 1(a)(iii)

```
            10          20          30       40
GAC ATC GTG ATG ACC CAG TCT CCT TCC ACC CTG TCT GCA TCT GTA GGA
 D   I   V   M   T   Q   S   P   S   T   L   S   A   S   V   G>

    50          60          70          80       90
GAC AGA GTC ACC ATC ACT TGC CGG GCC AGT CAG GGT ATT AGT AGC TGG
 D   R   V   T   I   T   C   R   A   S   Q   G   I   S   S   W>

100         110         120         130      140
TTG GCC TGG TAT CAG CAG AAA CCA GGG AGA GCC CCT AAG GTC TTG ATC
 L   A   W   Y   Q   Q   K   P   G   R   A   P   K   V   L   I>

    150         160         170         180      190
TAT AAG GCA TCT ACT TTA GAA AGT GGG GTC CCA TCA AGG TTC AGC GGC
 Y   K   A   S   T   L   E   S   G   V   P   S   R   F   S   G>

    200         210         220         230      240
AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGT CTG CAA CCT
 S   G   S   G   T   D   F   T   L   T   I   S   S   L   Q   P>

    250         260         270         280
GAA GAT TTT GCA ACT TAC TAC TGT CAA CAG AGT TAC AGT ACC CCG TGG
 E   D   F   A   T   Y   Y   C   Q   Q   S   Y   S   T   P   W>

290         300         310         320
ACG TTC GGC CAA GGG ACC AAG CTG GAG ATC AAA CGT
 T   F   G   Q   G   T   K   L   E   I   K   R
```

Figure 1(b)(i)

```
             10          20          30          40
GAC ATC GTG ATG ACC CAG TCT CCA GAC TCC CTG GCT GTG TCT CTG GGC
 D   I   V   M   T   Q   S   P   D   S   L   A   V   S   L   G >

  50          60          70          80          90
GAG AGG GCC ACC ATC AAC TGC AAG TCC AGC CAG AGT CTT TTA TAC AGC
 E   R   A   T   I   N   C   K   S   S   Q   S   L   L   Y   S >

 100         110         120         130         140
TAC AAC AAG ATG AAC TAC TTA GCT TGG TAC CAG CAG AAA CCA GGA CAG
 Y   N   K   M   N   Y   L   A   W   Y   Q   Q   K   P   G   Q >

 150         160         170         180         190
CCT CCT AAG CTG CTC ATT AAC TGG GCA TCT ACC CGG GAA TCC GGG GTC
 P   P   K   L   L   I   N   W   A   S   T   R   E   S   G   V >

 200         210         220         230         240
CCT GAC CGA TTC AGT GGC AGC GGG TCT GGG ACA GAT TTC ACT CTC ACC
 P   D   R   F   S   G   S   G   S   G   T   D   F   T   L   T >

 250         260         270         280
ATC AGC AGC CTG CAG GCT GAA GAT GTG GCA GTT TAT TAC TGT CAG CAA
 I   S   S   L   Q   A   E   D   V   A   V   Y   Y   C   Q   Q >

 290         300         310         320         330
TAT TAT GCA ACT CCT CTG ACG TTC GGC CAC GGG ACC AAG GTG GAA ATC
 Y   Y   A   T   P   L   T   F   G   H   G   T   K   V   E   I >

 340
AAA CGT
 K   R
```

Figure 1(b)(ii)

```
              10           20           30           40
CAC GTT ATA CTG ACT CAG CCT GCT GTG TCT GTG GCC TTG GGA CAG
 H   V   I   L   T   Q   P   A   V   S   V   A   L   G   Q>

 50          60           70           80           90
ACA GTC AGG ATC ACG TGC CAA GGA GAC AGC CTC AAA AGC TAC TAT GCA
 T   V   R   I   T   C   Q   G   D   S   L   K   S   Y   Y   A>

             100          110          120          130          140
AGT TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT
 S   W   Y   Q   Q   K   P   G   Q   A   P   V   L   V   I   Y>

             150          160          170          180          190
GGT GAA AAC AGC CGG CCC TCC GGG ATC CCA GAC CGA TTC TCT GGC TCC
 G   E   N   S   R   P   S   G   I   P   D   R   F   S   G   S>

             200          210          220          230          240
AGC TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAA
 S   S   G   N   T   A   S   L   T   I   T   G   A   Q   A   E>

             250          260          270          280
GAT GAA GCT GAC TAT TAC TGT TGC AAC TCC CGG GAC AGC AGT GGT ACC CAT
 D   E   A   D   Y   Y   C   C   N   S   R   D   S   S   G   T   H>

 290         300          310          320          330
CTA GAA GTG TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
 L   E   V   F   G   G   G   T   K   L   T   V   L   G
```

Figure 1(c)(i)

```
                  10                  20                  30                  40
CAG GTG CAA CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG
 Q   V   Q   L   V   E   S   G   G   G   V   V   Q   P   G   R>
 50                  60                  70                  80                  90
TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA CTC ACC TTC AGT AGC TAT
 S   L   R   L   S   C   A   A   S   G   L   T   F   S   S   Y>
100                 110                 120                 130         140
GAC ATG CAC TGG GTC CGC CAG CCT CCA GCC AAG GGG CTG GAG TGG GTG
 D   M   H   W   V   R   Q   P   P   A   K   G   L   E   W   V>
150                 160                 170                 180         190
GCA GTT ATA TCA TAT GAT GGA AGT AGT AAA TAC TAT GCA GAC TCC GTG
 A   V   I   S   Y   D   G   S   S   K   Y   Y   A   D   S   V>
200                 210                 220                 230         240
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
 K   G   R   F   T   I   S   R   D   N   S   K   N   T   L   Y>
250                 260                 270                 280
CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCT GTG TAT TAC TGT
 L   Q   M   N   S   L   R   A   E   D   T   A   V   Y   Y   C>
290                 300                 310                 320         330
GCG CGA ACT GGT GAA TAT AGT GGC TAC GAC AGT GGT GTG GAG CTC
 A   R   T   G   E   Y   S   G   Y   D   S   G   V   E   L>
340                 350                 360
TGG GGG CAA GGG ACC ACG GTC ACC GTC TCC TCA
 W   G   Q   G   T   T   V   T   V   S   S
```

Figure 2(a)(i)

```
                   10          20          30        40
GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG
 E   V   Q   L   V   E   S   G   G   G   V   V   Q   P   G   R>

50          60          70          80          90
TCC CTG AGA CTC TCC TGT GCA GCG TCT GGA TTC ACC TTC AGT AGC TAT
 S   L   R   L   S   C   A   A   S   G   F   T   F   S   S   Y>

100         110         120         130       140
GGC ATG CAC TGG GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG
 G   M   H   W   V   R   Q   A   P   G   K   G   L   E   W   V>

150         160         170         180       190
GCA GTT ATA TGG TAT GAT GGA AGT AAT AAA TAC TAT GCA GAC TCC GTG
 A   V   I   W   Y   D   G   S   N   K   Y   Y   A   D   S   V>

200         210         220         230       240
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
 K   G   R   F   T   I   S   R   D   N   S   K   N   T   L   Y>

250         260         270         280
CTG CAA ATG GAC AGC CTG AGA GCC GAG GAC ACG GCC GTG TAT TAC TGT
 L   Q   M   D   S   L   R   A   E   D   T   A   V   Y   Y   C>

290         300         310         320       330
GGA AGA ACG CTG GAG TCT AGT TTG TGG GGC CAA GGG ACC CTG GTC ACC
 G   R   T   L   E   S   S   L   W   G   Q   G   T   L   V   T>

340
GTC TCC TCA
 V   S   S
```

Figure 2(a)(ii)

```
                        10              20              30          40
GAG ATT CAG CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGA
 E   I   Q   L   V   E   S   G   G   G   V   V   Q   P   G   R>

50              60              70          80          90
TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT AGC TAT
 S   L   R   L   S   C   A   A   S   G   F   T   F   S   S   Y>

100             110             120         130         140
GCT ATG CAC TGG GTC CGC CAG GCT CCA GCC AAG GGG CTG GAG TGG GTG
 A   M   H   W   V   R   Q   A   P   A   K   G   L   E   W   V>

150             160             170         180         190
GCA GTT ATA TCA TAT GAT GGA AGC AAT AAA TAC TAC GCA GAC TCC GTG
 A   V   I   S   Y   D   G   S   N   K   Y   Y   A   D   S   V>

200             210             220         230         240
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
 K   G   R   F   T   I   S   R   D   N   S   K   N   T   L   Y>

250             260             270         280
CTG CAA ATG AAC AGC CTG AGA GCT GAG GAC ACG GCC GTG TAT TAC TGT
 L   Q   M   N   S   L   R   A   E   D   T   A   V   Y   Y   C>

290             300         310             320         330
GCA AGA GCG GGG TTG GAA ACG TGG GGC CAA GGA ACC CTG GTC ACC
 A   R   A   G   L   E   T   W   G   Q   G   T   L   V   T>

340             350
GTC TCC TCA AGT GG
 V   S   S   S   G
```

Figure 2(b)(i)

```
                 10              20              30              40
GAT GTT GTG ATG ACT CAG TCT CCA TCC CTG TCT GCA TCT GTA GGA
 D   V   V   M   T   Q   S   P   S   L   S   A   S   V   G>

 50             60              70              80              90
GAC AGA GTC ACC ATC ACT TGC CGG GCC AGT CAG GGC ATT AGC AAT TAT
 D   R   V   T   I   T   C   R   A   S   Q   G   I   S   N   Y>

100            110             120             130             140
TTA GCC TGG TAT CAG CAA AAA CCA GGG AAA GCC CCT AAG CTC CTG ATC
 L   A   W   Y   Q   Q   K   P   G   K   A   P   K   L   L   I>

150            160             170             180             190
TAT AAG GCA TCT ACT TTA GAA AGT GGG GTC CCA TCA AGG TTC AGT GGC
 Y   K   A   S   T   L   E   S   G   V   P   S   R   F   S   G>

200            210             220             230             240
AGT TCT GGG ACA GAA TTC ACT CTC ACA ATC AGC AGT CTG CAA CCT
 S   S   G   T   E   F   T   L   T   I   S   S   L   Q   P>

250            260             270             280
GAA GAT TTT GCA ACT TAC TAC TGT CAA CAG AGT TAC AGT ACC CCT CGA
 E   D   F   A   T   Y   Y   C   Q   Q   S   Y   S   T   P   R>

290            300             310             320             330
ACG TTC GGC CAA GGG ACC AAA GTG GAT ATC AAA CGT
 T   F   G   Q   G   T   K   V   D   I   K   R
```

Figure 2(b)(ii)

```
        10              20              30          40
TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG
 S   S   E   L   T   Q   D   P   A   V   S   V   A   L   G   Q>

50              60              70          80          90
ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT TAT GCA
 T   V   R   I   T   C   Q   G   D   S   L   R   S   Y   Y   A>

100             110             120         130         140
AGC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT
 S   W   Y   Q   Q   K   P   G   Q   A   P   V   L   V   I   Y>

150             160             170         180         190
GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC TTC GCT GGC TCC
 G   K   N   N   R   P   S   G   I   P   D   F   A   G   S>

200             210             220         230         240
AAC TCA GGA AAC ACA GCT GGA AAC ATC ACT GGG GCT CAG GCG GAG
 N   S   G   N   T   A   G   N   I   T   G   A   Q   A   E>

250             260             270         280
GAT GAG GCT GAC TAT TAC TGT AGC TCC CGG GAC AGC AGT GGT AAC CAT
 D   E   A   D   Y   Y   C   S   S   R   D   S   S   G   N   H>

290             300             310         320
GTG GTT TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
 V   V   F   G   G   G   T   K   L   T   V   L   G>
```

Figure 2(b)(iii)

```
          10          20          30          40
TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG
 S   S   E   L   T   Q   D   P   A   V   S   V   A   L   G   Q>

 50          60          70          80          90
ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT TAT GCA
 T   V   R   I   T   C   Q   G   D   S   L   R   S   Y   Y   A>

100         110         120         130         140
AGC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT
 S   W   Y   Q   Q   K   P   G   Q   A   P   V   L   V   I   Y>

150         160         170         180         190
GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC
 G   K   N   N   R   P   S   G   I   P   D   R   F   S   G   S>

200         210         220         230         240
AGC TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAA
 S   S   G   N   T   A   S   L   T   I   T   G   A   Q   A   E>

250         260         270         280
GAT GAG GCT GAC TAT TAC TGT AAC TCC CGG GAC AGC AGT AGT ACC CAT
 D   E   A   D   Y   Y   C   N   S   R   D   S   S   S   T   H>

290         300         310         320         330
CGA GGG GTG TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
 R   G   V   F   G   G   G   T   K   L   T   V   L   G
```

Figure 2(b)(iv)

```
          10          20              30              40
GAA GTT GTG CTG ACT CAG TCT CCA TCC CTG TCT GCA TCT GTA GGA
 E   V   V   L   T   Q   S   P   S   L   S   A   S   V   G>

 50              60          70              80          90
GAC AGA GTC ATC ACT TGC CGG GCA AGT CAG GGC ATT GGA GAT GAT
 D   R   V   I   T   C   R   A   S   Q   G   I   G   D   D>

100         110         120         130         140
TTG GGC TGG TAT CAG CAG AAG CCA GGG AAA GCC CCT ATC CTC ATC
 L   G   W   Y   Q   Q   K   P   G   K   A   P   I   L   I>

150         160         170         180         190
TAT GGT ACA TCC ACT TTA CAA AGT GGG GTC CCG TCA AGG TTC AGC GGC
 Y   G   T   S   T   L   Q   S   G   V   P   S   R   F   S   G>

200         210         220         230         240
AGT GGA TCT GGC ACA GAT TTC ACT CTC ACC ATC AAC AGC CTG CAG CCT
 S   G   S   G   T   D   F   T   L   T   I   N   S   L   Q   P>

250         260         270         280
GAA GAT TTT GCA ACT TAT TAC TGT CTA CAA GAT TCC AAT TAC CCG CTC
 E   D   F   A   T   Y   Y   C   L   Q   D   S   N   Y   P   L>

290         300         310         320
ACT TTC GGC GGA GGG ACA CGA CTG GAG ATT AAA CGT
 T   F   G   G   G   T   R   L   E   I   K   R
```

Figure 2(b)(v)

```
                    10              20              30          40
TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG
 S   S   E   L   T   Q   D   P   A   V   S   V   A   L   G   Q>

 50             60              70          80              90
ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AAC TAT TAT GCA
 T   V   R   I   T   C   Q   G   D   S   L   R   N   Y   Y   A>

100             110             120         130             140
AAC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT
 N   W   Y   Q   Q   K   P   G   Q   A   P   V   L   V   I   Y>

150             160             170         180             190
GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC
 G   K   N   N   R   P   S   G   I   P   D   R   F   S   G   S>

200             210             220         230             240
AGC TCA GGG AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CGG GCG GAA
 S   S   G   N   T   A   S   L   T   I   T   G   A   R   A   E>

250             260         270             280
GAT GAG GGT GTC TAT TAC TGT AAC TCC CGG GAC AGC AGT GCG GTT
 D   E   G   V   Y   Y   C   N   S   R   D   S   S   A   V>

290             300         310             320
TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
 F   G   G   G   T   K   L   T   V   L   G
```

PARENT (1-B2)	A R T G E Y S G Y D S S G V D V W
27-C1	A R T G E Y S G Y D T S G V E L W
27-D7	A R T R E Y S G H D S S G V D D W
27-E10	A R T G P F S G Y D S S G E D V R
27-H1	A R T E E Y S G Y D S S G V D V W
27-E2	A Q T R E Y T G Y D S S G V D V W
28-A11	A R T E E Y S G F D S T G E D V W
28-E12	A R T E E F S G Y D S S G V D V W
28-H10	A R T G E Y S G Y H S S G V D V R
31-G2	A R T E E F S G Y D S S G V D V W
30-B6	A R A G P F S G Y D S S G E D V R
30-E9	A R T G P F S G Y D S S G E D V W
30-F6	A R T E E F S G Y D S S G V D V W
30-D2	A R T G E Y S G Y D S S G E L V W
31-A2	A R T E E F S G Y D S T G E E V W
31-E11	A R T E E F S G Y D S S G V D V W
31-F1	A R T G E Y S G Y D S S G E D V W

Figure 4

```
        10          20          30          40
TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG
 S   S   E   L   T   Q   D   P   A   V   S   V   A   L   G   Q>

50          60          70          80          90
ACA GTT AGG ATC ACT TCC CAA GGA GAC AGT CTC AGA AGC TAT TAC ACA
 T   V   R   I   T   S   Q   G   D   S   L   R   S   Y   Y   T>

100         110         120         130         140
AAC TGG TTT CAG CAG AAG CCA GGA CAG CCC CCT CTA CTT GTC GTC TAT
 N   W   F   Q   Q   K   P   G   Q   P   P   L   L   V   V   Y>

150         160         170         180         190
GCT AAA AAT AAG CGG CCC TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC
 A   K   N   K   R   P   S   G   I   P   D   R   F   S   G   S>

200         210         220         230         240
AGC TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAA
 S   S   G   N   T   A   S   L   T   I   T   G   A   Q   A   E>

250         260         270         280
GAT GAG GCT GAC TAT TAC TGT CAT TCC CGG GAC AGC AGT GGT AAG CAT
 D   E   A   D   Y   Y   C   H   S   R   D   S   S   G   K   H>

290         300         310         320
GTG CTT TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
 V   L   F   G   G   G   T   K   L   T   V   L   G
```

```
        H
        i
        n
        d
        I
        I
        I
        aagcttgccgccaccatggactggacctggcgcgtgttttgcctgctcgccgtggcccct
   1    --------+---------+---------+---------+---------+---------+  60
        ttcgaacggcggtggtacctgacctggaccgcgcacaaaacggacgagcggcaccgggga

a       K  L  A  A  T  M  D  W  T  W  R  V  F  C  L  L  A  V  A  P  -
                                                              B
                                                              s
        s                       P                             t
        f                       s                             E
        i                       t                             I
        I                       I                             I
        ggggcccacagccaggtgcaactgcagcagtccggtgccaagggaccacggtcaccgtct
  61    --------+---------+---------+---------+---------+---------+  120
        ccccgggtgtcggtccacgttgacgtcgtcaggccacggttccctggtgccagtggcaga

a       G  A  H  S  Q  V  Q  L  Q  Q  S  G  A  K  G  P  R  S  P  S  -
                       B         E
                       a         c
                       m         o
                       H         R
                       I         I
        cctcaggtgagtggatccgaattc
 121    --------+---------+----  144
        ggagtccactcacctaggcttaag

a       P  Q  V  S  G  S  E  F  -
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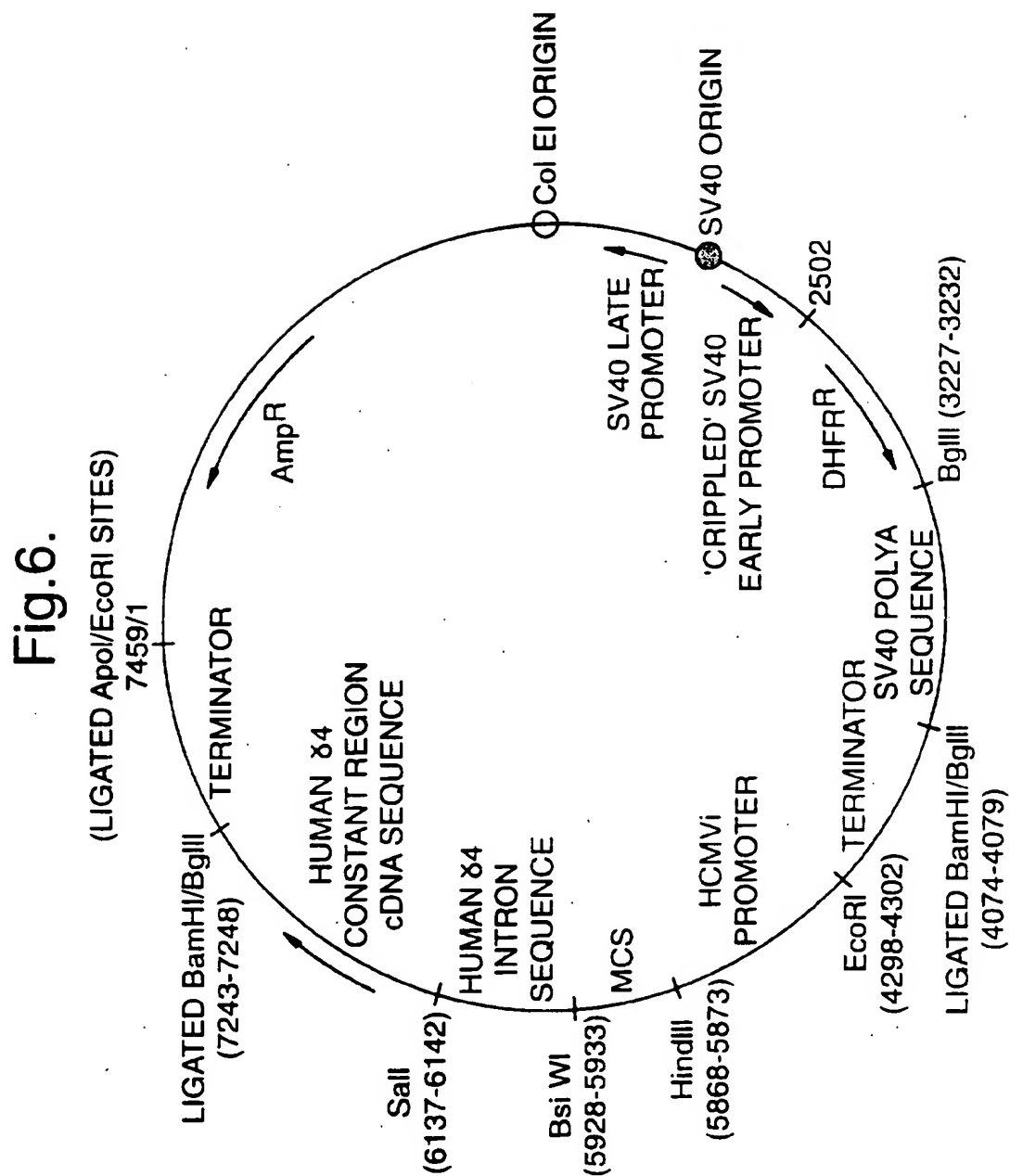
Fig.6.

```
H
i
n
d
I
I
I
aagcttcgccaccatgggatggagctgtatcatcctcttcttggtagcaacagctacagg
1  ---------+---------+---------+---------+---------+---------+ 60
ttcgaagcggtggtaccctacctcgacatagtaggagaagaaccatcgttgtcgatgtcc

        M  G  W  S  C  I  I  L  F  L  V  A  T  A  T

taaggggctcacagtagcaggcttgaggtctggacatatatatgggtgacaatgacatcc
61 ---------+---------+---------+---------+---------+---------+ 120
attccccgagtgtcatcgtccgaactccagacctgtatatatacccactgttactgtagg

                    A                         S
                    p                         a
                    a                         c
                    L                         I
                    I
actttgcctttctctccacaggtgtgcactccgacattgagctcacccagtctccagaca
121 --------+---------+---------+---------+---------+---------+ 180
tgaaacggaaagagaggtgtccacacgtgaggctgtaactcgagtgggtcagaggtctgt

                    G  V  H  S  D  I  E  L

                                              B
                                              a
        X                                     m
        h                                     H
        o                                     I
        I
aagctcgagctgaaacgtgagtagaatttaaactttgcttcctcaattggatcc
181 --------+---------+---------+---------+---------+---- 234
ttcgagctcgactttgcactcatcttaaatttgaaacgaaggagttaacctagg

    L  E  L  K
```

Fig.8.

EcoRI (1-6)
7121/1

TERMINATOR SEQUENCE

HCMV PROMOTER

HindIII (1224-1229)

MCS

BamHI (1254-1259)

INTRON OF HUMAN KAPPA CONSTANT REGION

Bsu 36 I (1375-1381)

HUMAN LAMBDA CONSTANT REGION SEQUENCE (cDNA CLONE)

LIGATED BamHI/BglII (1711-1716)

TERMINATOR SEQUENCE

EcoRI (1934-1939)

AmpR

ColEI ORIGIN OF REPLICATION

SV40 ORIGIN OF REPLICATION

SV40 LATE PROMOTER

SV40 EARLY PROMOTER

NeoR

LIGATED BamHI/BglII (6899-6905)

Fig.9.

Fig.10.

Fig.11.

Fig.12.

Fig.13(a).

Fig.13(b).

Figure 14


```
              10          20          30          40
GAA ATT GTG CTG ACT CAG TCT CCA TCC CTG TCT GCA TCT GTA GGA
 E   I   V   L   T   Q   S   P   S   L   S   A   S   V   G>

 50          60          70          80          90
GAC AGA GTC ACC ATC ACT TGC CGG GCA AGT CAG GGC ATT GGA GAT GAT
 D   R   V   T   I   T   C   R   A   S   Q   G   I   G   D   D>

100         110         120         130         140
TTG GGC TAT CAG CAG AAG CCA GGG AAA GCC CCT ATC CTC CTG ATC
 L   G   Y   Q   Q   K   P   G   K   A   P   I   L   L   I>

150         160         170         180         190
TAT GGT ACA TCC TTA CAA AGT GGG GTC CCG TCA AGG TTC AGC GGC
 Y   G   T   S   L   Q   S   G   V   P   S   R   F   S   G>

200         210         220         230         240
AGT GGA TCT GGC ACA GAT TTC ACT CTC ACC ATC AAC AGC CTG CAG CCT
 S   G   S   G   T   D   F   T   L   T   I   N   S   L   Q   P>

250         260         270         280
GAA GAT TTT GCA ACT TAT TAC TGT CTA CAA GAT TCC AAT TAC CCG CTC
 E   D   F   A   T   Y   Y   C   L   Q   D   S   N   Y   P   L>

290         300         310         320
ACT TTC GGC GGA GGG ACA CGA CTG GAG ATT AAA CGT
 T   F   G   G   G   T   R   L   E   I   K   R>
```
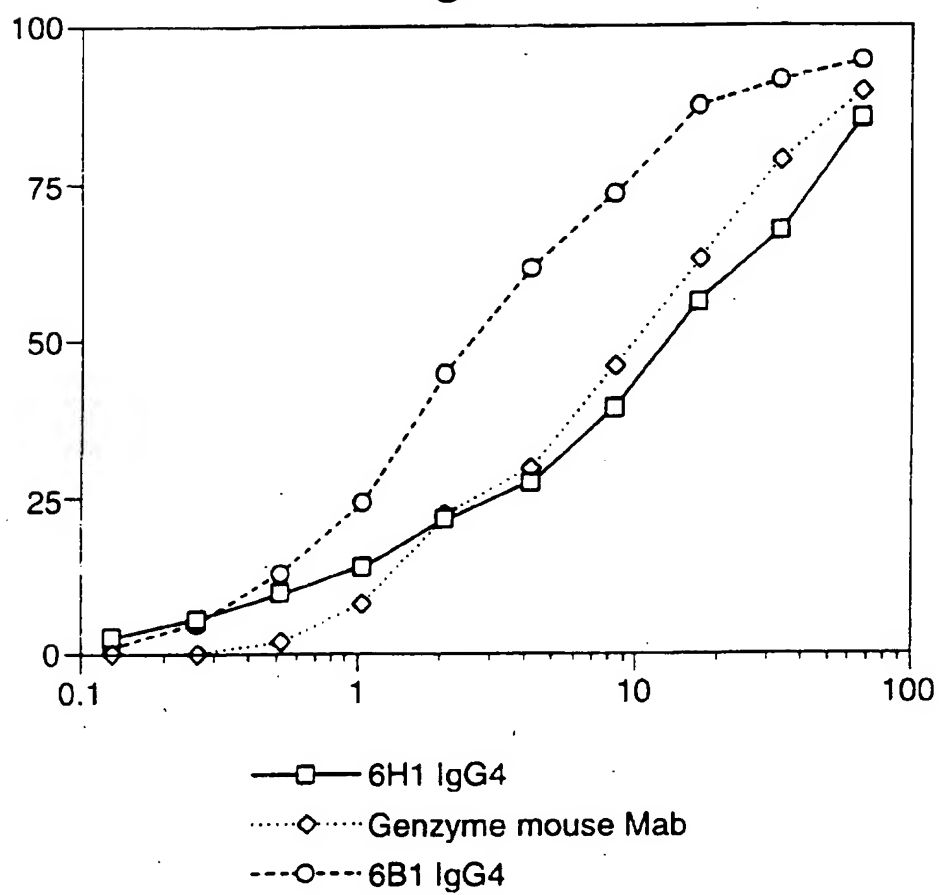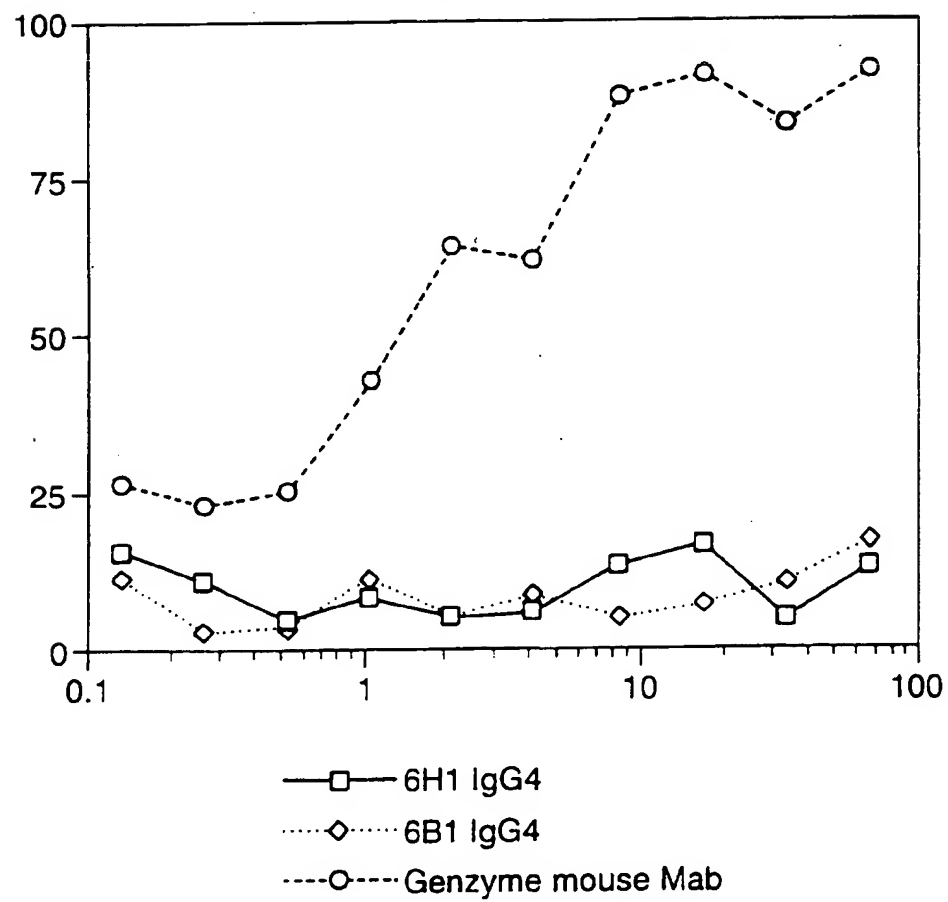
Fig.15.

Fig.16.

—□— 6H1 IgG4

·····◇····· Genzyme mouse Mab

---○--- 6B1 IgG4

Fig.17.

—□— 6H1 IgG4

·····◇····· 6B1 IgG4

----O---- Genzyme mouse Mab

Fig.18.

— —□— 6H1 IgG4

·····◇····· 6B1 IgG4

- - -○- - - Genzyme mouse Mab
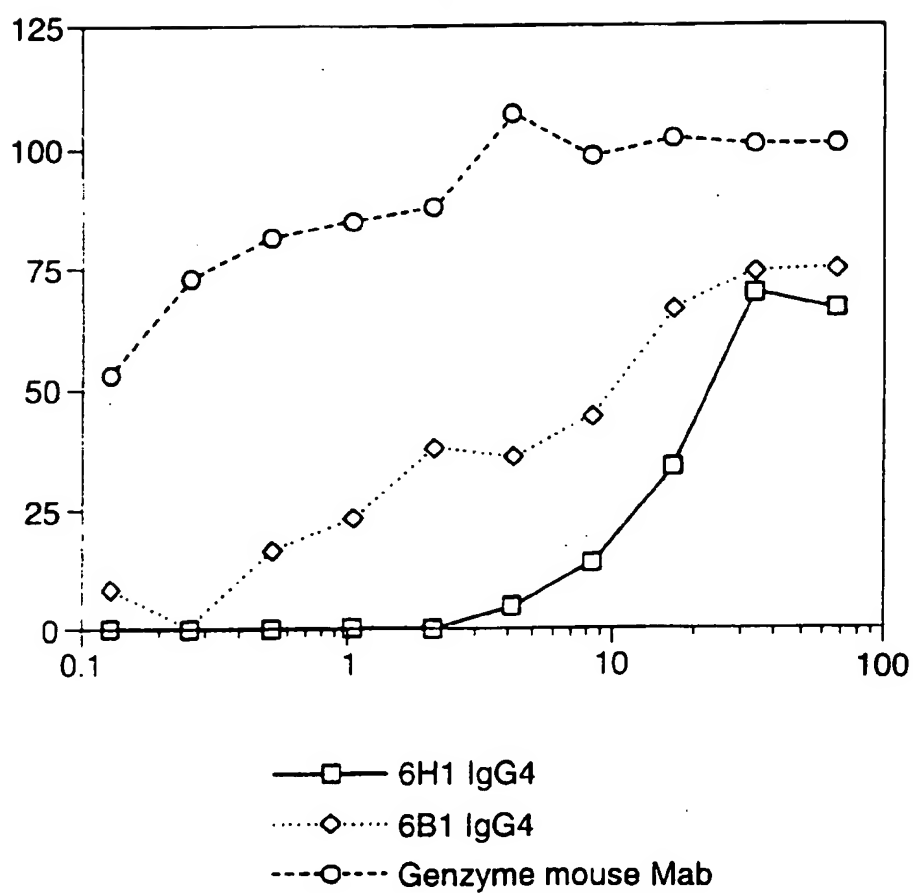
Figure 19

(i)

```
        10          20          30       40
GAG GTG CAG CTG GTG GAG TCT GGG GGA GGC GTG GTC CAG CCT GGG AGG
 E   V   Q   L   V   E   S   G   G   G   V   V   Q   P   G   R>

 50          60          70       80          90
TCC CTG AGA CTC TCC TGT GCA GCG TCT GGA TTC ACC TTC AGT AGC TAT
 S   L   R   L   S   C   A   A   S   G   F   T   F   S   S   Y>

100         110         120      130         140
GGC ATG CAC TGG GTC CGC CAG GCT CCA GGC AAG GGG CTG GAG TGG GTG
 G   M   H   W   V   R   Q   A   P   G   K   G   L   E   W   V>

150         160         170      180         190
GCA GTT ATA TGG TAT GAT GGA AGT AAT AAA TAC TAT GCA GAC TCC GTG
 A   V   I   W   Y   D   G   S   N   K   Y   Y   A   D   S   V>

200         210         220      230         240
AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT TCC AAG AAC ACG CTG TAT
 K   G   R   F   T   I   S   R   D   N   S   K   N   T   L   Y>

250         260         270      280
CTG CAA ATG GAC AGC CTG AGA GCC GAG GAC ACG GCC GTG TAT TAC TGT
 L   Q   M   D   S   L   R   A   E   D   T   A   V   Y   Y   C>

290         300         310      320         330
GGA AGA ACG CTG GAG TCT AGT TTG TGG GGC CAA GGG ACC CTG GTC ACC
 G   R   T   L   E   S   S   L   W   G   Q   G   T   L   V   T>

340
GTC TCC TCA
 V   S   S
```

Figure 19 (ii)

```
        10              20              30              40
TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG
 S   S   E   L   T   Q   D   P   A   V   S   V   A   L   G   Q>

 50              60              70              80              90
ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT TAT GCA
 T   V   R   I   T   C   Q   G   D   S   L   R   S   Y   Y   A>

 100             110             120             130             140
AGC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT
 S   W   Y   Q   Q   K   P   G   Q   A   P   V   L   V   I   Y>

 150             160             170             180             190
GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC
 G   K   N   N   R   P   S   G   I   P   D   R   F   S   G   S>

 200             210             220             230             240
AGC TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAA
 S   S   G   N   T   A   S   L   T   I   T   G   A   Q   A   E>

 250             260             270             280
GAT GAG GCT GAC TAT TAC TGT AAC TCC CGG GAC AGC AGT AGT ACC CAT
 D   E   A   D   Y   Y   C   N   S   R   D   S   S   S   T   H>

 290             300             310             320             330
CGA GGG GTG TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
 R   G   V   F   G   G   G   T   K   L   T   V   L   G
```

Figure 19 (iii)

```
          10              20              30          40
TCG TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG
 S   S   E   L   T   Q   D   P   A   V   S   V   A   L   G   Q>

  50              60              70          80          90
ACA GTC AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT TAT GCA
 T   V   R   I   T   C   Q   G   D   S   L   R   S   Y   Y   A>

 100             110             120         130         140
AGC TGG TAC CAG CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT
 S   W   Y   Q   Q   K   P   G   Q   A   P   V   L   V   I   Y>

 150             160             170         180         190
GGT AAA AAC AAC CGG CCC TCA GGG ATC CCA GAC CGA TTC GCT GGC TCC
 G   K   N   N   R   P   S   G   I   P   D   R   F   A   G   S>

 200             210             220         230         240
AAC TCA GGA AAC ACA GCT TCC TTG ACC ATC ACT GGG GCT CAG GCG GAG
 N   S   G   N   T   A   S   L   T   I   T   G   A   Q   A   E>

 250             260             270         280
GAT GAG GCT GAC TAT TAC TGT AGC TCC CGG GAC AGC AGT GGT AAC CAT
 D   E   A   D   Y   Y   C   S   S   R   D   S   S   G   N   H>

 290             300             310         320
GTG GTT TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA GGT
 V   V   F   G   G   G   T   K   L   T   V   L   G
```

Figure 19(iv)

```
        10            20            30            40
GAT GTT GTG ATG ACT CAG TCT CCA TCC CTG TCT GCA TCT GTA GGA
 D   V   V   M   T   Q   S   P   S   L   S   A   S   V   G>

        60            70            80            90
GAC AGA GTC ACC ATC ACT TGC CGG GCC AGT CAG GGC ATT AGC AAT TAT
 D   R   V   T   I   T   C   R   A   S   Q   G   I   S   N   Y>

100           110           120           130           140
TTA GCC TGG TAT CAG CAA AAA CCA GGG AAA GCC CCT AAG CTC CTG ATC
 L   A   W   Y   Q   Q   K   P   G   K   A   P   K   L   L   I>

150           160           170           180           190
TAT AAG GCA TCT ACT TTA GAA AGT GGG GTC CCA TCA AGG TTC AGT GGC
 Y   K   A   S   T   L   E   S   G   V   P   S   R   F   S   G>

200           210           220           230           240
AGT GGA TCT GGG ACA GAA TTC ACT CTC ACA ATC AGC AGT CTG CAA CCT
 S   G   S   G   T   E   F   T   L   T   I   S   S   L   Q   P>

250           260           270           280
GAA GAT TTT GCA ACT TAC TAC TGT CAA CAG AGT TAC AGT ACC CCT CGA
 E   D   F   A   T   Y   Y   C   Q   Q   S   Y   S   T   P   R>

290           300           310           320
ACG TTC GGC CAA GGG ACC AAA GTG GAT ATC AAA CGT
 T   F   G   Q   G   T   K   V   D   I   K   R
```
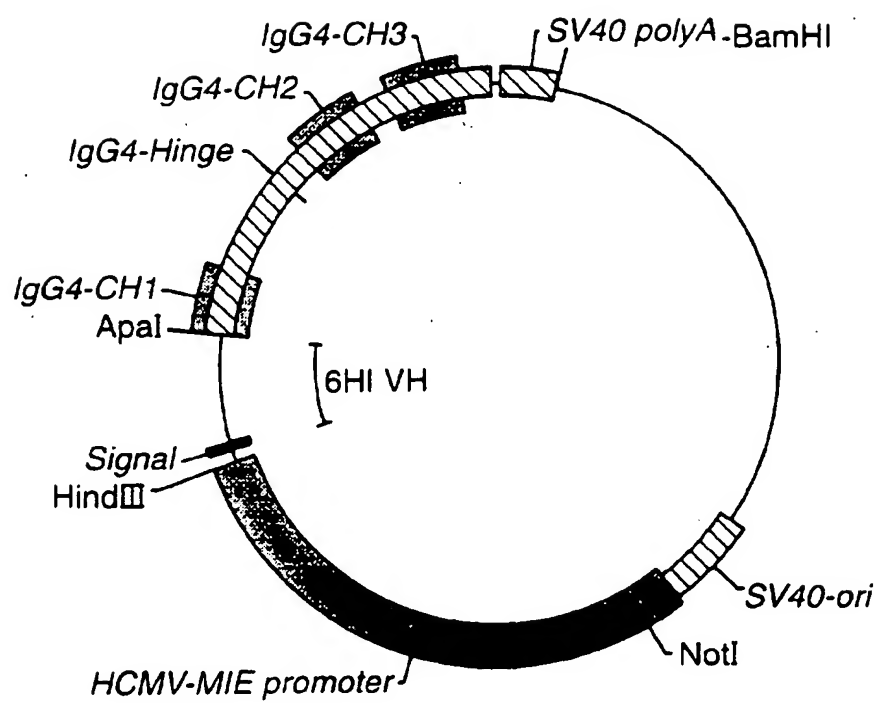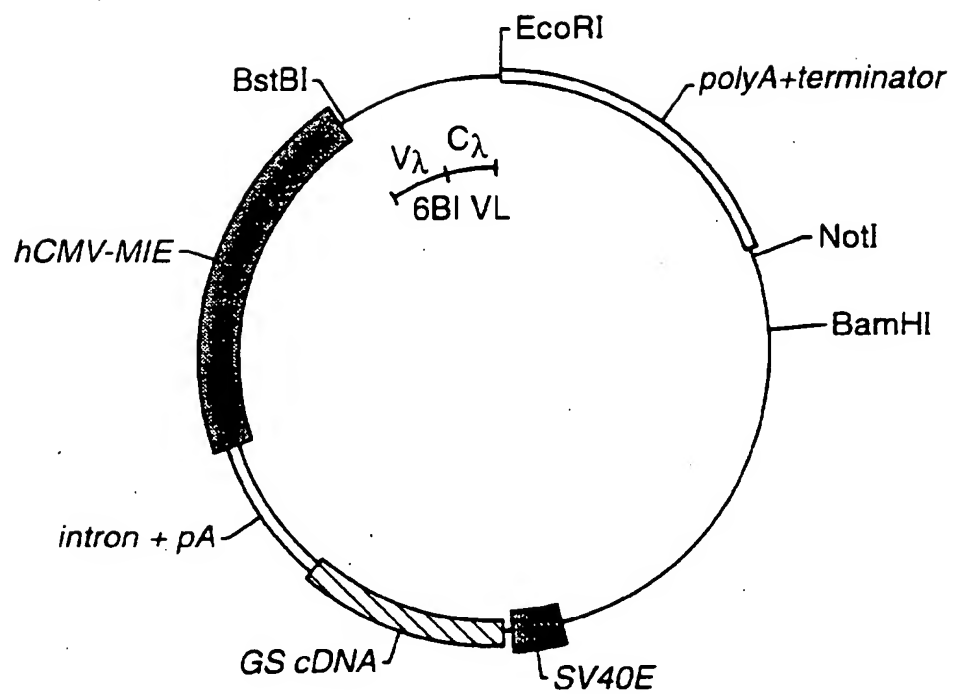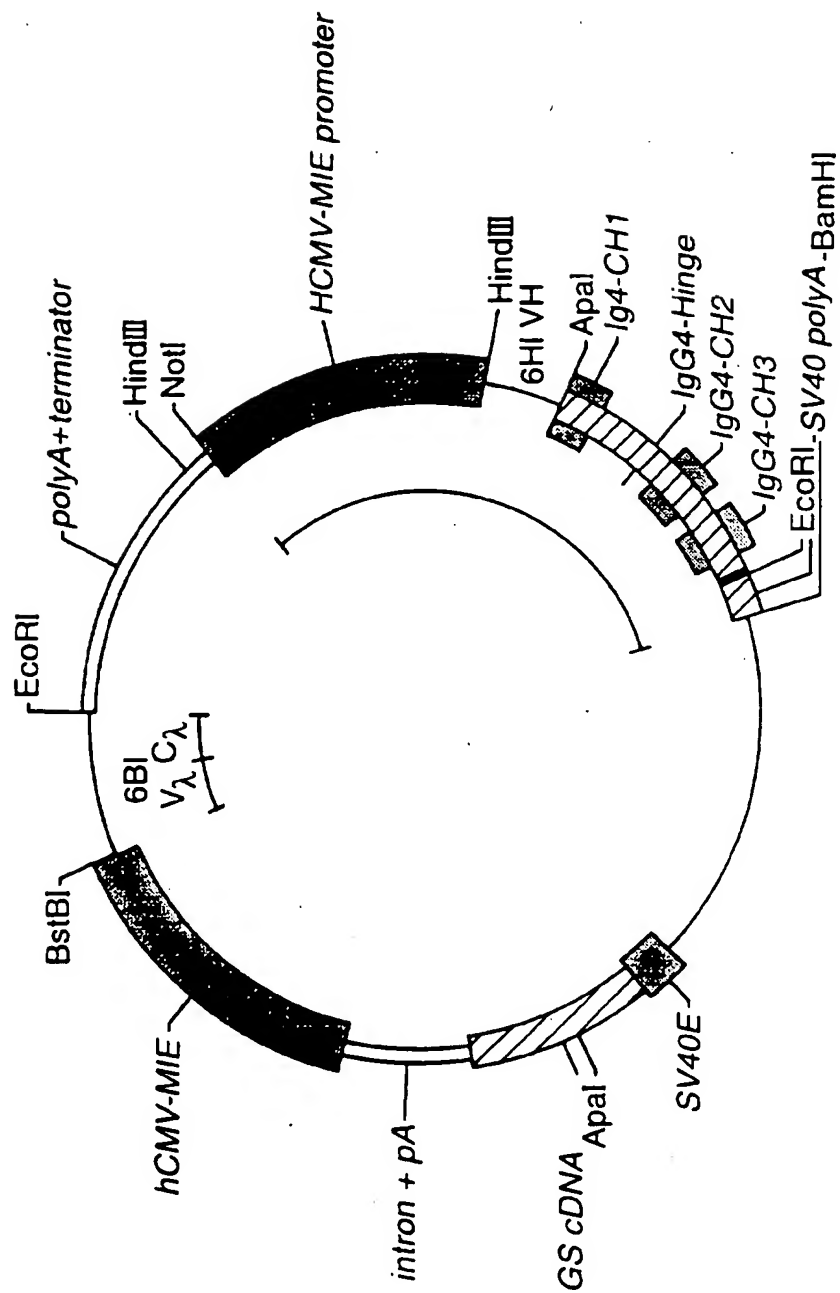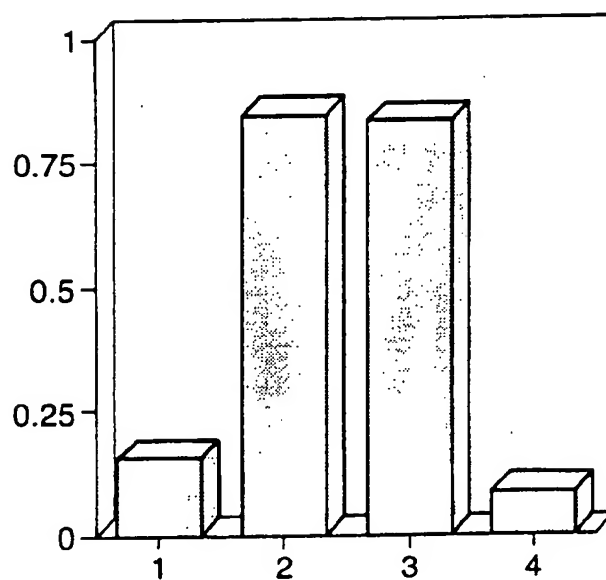
Fig.20.

Fig.21.

Fig.22.

Fig.23.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N5/10   C12N15/13   C07K16/22   A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N   C07K   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 21945 A (UNIV CALIFORNIA ;JOLLA CANCER RES FOUND (US)) 11 November 1993 see abstract and page 6 line6 -line 23 ---	1,2, 31-33, 35-41, 43, 54-57,59
X	WO 92 17206 A (UNIV MANCHESTER) 15 October 1992 cited in the application see claims ---	1-3, 31-33, 35-41, 54-57
	-/--	

[X] Further documents are listed in the continuation of box C.	[X] Patent family members are listed in annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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Date of the actual completion of the international search	Date of mailing of the international search report
17 February 1997	2 6. 02. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Müller, F

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